REMARKS

Claims 54, 55, 57-60, 81-85, 88, 94, 96, 100, 107, 115-117, and 119-123 are pending. Applicants have amended claims 54, 55, 57, 60, and 122, canceled claims 56, 111-114, and 118, and added new claim 123. Thus, in total, claims 1-53, 56, 61-80, 86, 87, 89-93, 95, 97-99, 101-106, and 108-114, and 118 have been canceled.

The amendments to claim 54 are supported throughout the specification, e.g., at page 3, line 21 ("protective immune response"), page 2, line 14 ("wild-type sequences"), page 4, line 6 ("human"), page 6, lines 1-8 ("protection against current or future HIV (e.g., HIV-1) infection"), page 14, lines 11 to 15 ("a DNA composition can include genes encoding an antigen from one clade A isolate, one clade B isolate, one clade C isolate, and one clade E isolate"), page 21, line 2 ("nucleic acids encoding two, three, four, five, or six distinct HIV Env gps"), and in, e.g., Examples 13 to 16. Claim 57 has been amended to depend from claim 54. Claim 60 has been amended to include language originally present in the claim, but erroneously omitted in a part amendment. Claim 122 has been amended to correct its dependency. Support for new claim 123 can be found throughout the specification, for example, at page 6, line 27, to page 7, line 2, and in Examples 13 to 16. Thus, the amendments and new claim 123 present no new matter.

Applicants acknowledge the Examiner's withdrawal of his prior rejections under 35 U.S.C. § 103.

35 U.S.C. § 112, First Paragraph

Claims 54-60, 81-85, 88, 94, 96, 100, 107 and 111-122 have been rejected as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

According to the Office, the newly amended scope "...(a) at least three and no more than five sets of nucleic acid molecules encoding HIV envelope glycoproteins, ..." (Claim 54); "...at least three different clades" (Claim 111) and "...at least four different clades" of Claims 111-114) are new matter" (Office Action at page 2). The Office concedes that "the specification

exemplified a method of using multiple sets of HIV *env* DNAs in Examples," but then argues that "the specification has not explicitly excluded any specific combinations of HIV *env* DNAs, such as a combination of two or more than five *env* DNAs from different clades. Thus, there is no support for the new limitation of "at least three and no more than five sets of nucleic acid molecules encoding HIV envelope glycoproteins" in the specification" (Office Action at page 3).

Applicants respectfully disagree, and note that the claims as presented in the last response were fully supported by the specification as filed. However, in the interests of moving this application towards allowance, applicants have amended claim 54 to further clarify the invention. Claim 54 now recites at least four different specific clades (A, B, C, and E), and this language is clearly supported in the originally filed application as detailed below. Applicants have canceled claims 111-114 in view of the amendment to claim 54.

Applicants have amended claim 54 to include the language:

> (a) at least four sets of nucleic acid molecules encoding wild-type HIV envelope glycoproteins, wherein each of the sets of nucleic acid molecules encodes an envelope glycoprotein of a different primary isolate including at least a clade A primary isolate, a clade B primary isolate, a clade C primary isolate, and a clade E primary isolate, ...

This language is supported in the application, e.g., at page 2, line 14 ("wild-type sequences"), and at page 6, line 26 ("'4-valent' ... refer[s] to [a] composition with 4 ... unique antigens"), page 14, lines 11 to 15 ("a DNA composition can include genes encoding an antigen from one clade A isolate, one clade B isolate, one clade C isolate, and one clade E isolate"), and in, e.g., Example 7, 10, and 13 to 16. In particular, applicants have amended claim 54 to cover the specific vaccine composition referred to in the application as DP6-001, which includes nucleic acid molecules that encode different HIV-1 Env glycoprotein antigens from clades A, B, C, and E, and a gag antigen from clade C (see, e.g., page 6, last paragraph).

Thus, applicants respectfully request that the Examiner reconsider and withdraw the rejections under 35 U.S.C. § 112, first paragraph.

35 U.S.C. § 103

The Office rejected claims 54-60, 81-85, 88, 94, 95, 100, 107, and 111-122 as allegedly unpatentable over Barnett et al. (*Vaccine*, 8:869-873, 1997; "Barnett"), Nabel et al., No. 02/032943 ("Nabel"), Gao et al. (*J. Virol.*, 70(3): 1651-1667, 1996, "Gao 1"), Gao, F. et al., Meeting Abstract, AIDS Vaccine, Abstract No. 201, 2001, "Gao 3"), Yoshida et al., Clin. Exp. Immunol., 124: 445-452 ("Yoshida"), and Evans et al., Vaccine, 15:2080-91 (2001) ("Evans"). Applicants traverse this rejection for the following reasons.

According to the Office Action (at page 4), Barnett discloses a method of inducing immune responses using "priming immunization with DNA plasmid vaccine containing envelope genes of primary strains, HIV-1_{US4} (clade B) and HIV-1_{CM235} (clade E), and boosted with their proteins. Both humoral and cell-mediated immune responses were tested." Barnett is also said to describe "that the DNA prime/subunit protein boost may be a safe and less costly alternative vaccination strategy because of the ability of HIV DNA vaccines to effectively and reproducibly induce immune responses."

However, the Office also concedes (at pages 4-5) that "Barnett does not teach the use of multiple HIV envelope DNAs and proteins of different clades as immunogens," and that "Barnett does not explicitly teach the following embodiments in the method: (1) a set of gag DNAs (Claim 54); (2) one of [sic, or] more of the sets of DNAs comprises optimized codons (Claims 96 and 120); (3) BaL isolate (Claims 115-117); Czm isolate (Claim 119) and adjuvant QS-21 (Claim 122). Thus, the Office cites all of the remaining references to make up for these deficiencies in Barnett.

In particular, the Office alleges that Nabel discloses a method of inducing an immune response against HIV in a mammal using sets of DNA encoded HIV envelope proteins of clade A, C, and E and a panel of DNA encoded HIV gag, and the use of optimized codons. Nabel is also said to show that the DNA constructs encoding HIV env and gag proteins can induce CTL and antibody responses in mice.

The Office cites Gao 1 for allegedly suggesting the use of a panel of envelope gene constructs from HIV-1 primary isolates of clade A to G for AIDS vaccine development to target

against a broader spectrum of viruses. The isolate 92US715 is said to be isolate B715 of the applicants' claim 94.

The Office cites Gao 3 for disclosing "the use of codon-usage optimized gag and env genes of a prototypic HIV-1 subtype C strain (96ZM651), which is same Czm isolate of the instant Claim 119, to improve HIV-1 protein expression in the context of a DNA vaccine" (Office Action at page 5). Gao 3 is said to describe that codon optimization increases HIV-1 gene expression in vitro and appears to enhance both humoral and cellular immune responses upon DNA vaccination in vivo. Thus, Gao 3 is relevant, if at all, only to claims that recite the Czm isolate and the use of codon-optimization, i.e., claims 96, 119, 120, and 123.

Next, the Office cites Yoshida as disclosing "a method of inducing immune response to HIV in a mammal using DNA encoding HIV envelope from BaL isolate" (at Office Action at pages 5-6). Thus, Yoshida is relevant only to claims 115-117 and 123.

In addition, the Office cites Evans for the use of adjuvant QS-21 to enhance immunogenicity of gp120 HIV-1MN protein immunization in a mammal (Office Action at page 6). Thus, Evans is of interest only with respect to dependent claim 122, which recites the QS-21 adjuvant.

Based on a combination of all of these secondary references with Barnett, the Office concludes (at pages 6 and 7 of the Office Action):

> It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the methods of Barnett, Nabel, Gao and Yoshida in order to increase the breadth of reactivity of a HIV vaccine cross genetic clades and increase the immunogenicity of the vaccine. One would have been motivated to do so, given the suggestion by Gao [1] that envelope genes from HIV-1 Clades A to G should prove valuable for AIDS vaccine development efforts targeted against a broader spectrum of viruses. There would have been a reasonable expectation of success that the polyvalent vaccine and codon optimized immunogen would generate enhanced immune responses, given the knowledge taught by Gao F. [Gao 3] that codon optimized DNA vaccines have higher expression level in vivo and result in increased immunogenicity of DNA vaccines.
>
> * * *
>
> Since the instant invention is drawn to combining some envelope genes and proteins of known HIV isolates to increase immune responses in a mammal,

> the combination of their additive effects renders the invention *prima facie* obvious and does not exhibit an unexpected result. Thus, the invention as a whole was clearly *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

The Office then further concludes (at pages 7 to 8 of the Action):

> According to M.P.E.P. § 2143.02, "Obviousness does not require absolute predictability, however at least some degree of predictability is required. Evidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness. *In re Rinehart*, 531 F2d 1048, 189 USPQ 143 (CCPA 1976)." In the present case, the cited reference[s] have taught one of skill in the art that the alleged method steps: DNA priming and protein booting immunization, and all the compositions of HIV env and gag. The cited references have explicitly suggested to use a combination of HIV Clades A to G as multiple (or polyvalent) vaccine composition. The cited references have also shown that one of ordinary skill in the art is capable of testing vaccine compositions, either 2-valent or multiple 1-valent compositions, in animals. Thus, it is within the knowledge of one of ordinary skill in the art to optimize specific combinations of multiple HIV strains as [an] alleged vaccine using routine laboratory practice. Moreover, Examples 8 and 9 have shown that in rabbits, both 3-valent and 8-valent compositions can induce antibody responses, which is consistent with the teachings of the prior art. Thus, *Applicant has not presented any evidence showing that there was no reasonable expectation of success in using the approach and the compositions taught by the prior art references*. (emphasis added)

Applicants respectfully submit that the present claim amendments more clearly define the invention. In addition, applicants discuss below and submit herewith new evidence in the form of a journal article (Wang et al., *Vaccine,* 26:3947-3957 (2008)(copy provided as Exhibit A) that summarizes applicants' human clinical testing of the claimed methods. This article provides exactly the type of evidence that the Examiner seeks in the obviousness rejection quoted above. Applicants request that the Examiner consider the amended claims, the following remarks, and the new evidence and withdraw the rejection under Section 103.

First, applicants review the presently claimed invention of claim 54. This claim now recites (new language underlined):

> A method of inducing a protective immune response against human immunodeficiency virus (HIV) or an HIV epitope in a human, the method comprising:
>
> administering to the human a nucleic acid composition comprising (a) at least four sets of nucleic acid molecules encoding wild-type HIV envelope glycoproteins, wherein each of the sets of nucleic acid molecules encodes an envelope glycoprotein of a different primary isolate including at least a clade A primary isolate, a clade B primary isolate, a clade C primary isolate, and a clade E primary isolate, and (b) a set of nucleic acid molecules encoding [an] a wild-type HIV gag protein of a primary isolate of clade C; and
>
> thereafter administering to the human a protein composition comprising a plurality of sets of isolated wild-type HIV envelope glycoprotein molecules of each of the primary isolates in (a),
>
> wherein the nucleic acid composition and the protein composition are administered in amounts sufficient to elicit [[an]] a protective immune response against a current or future infection with HIV or an HIV epitope in the human.

Thus, elements of claim 54 now include that (i) the method provides not just an immune response, but a "protective immune response" (as defined in the application) against a current or future HIV infection in a human, (ii) the nucleic acid molecules encode "wild-type" HIV envelope glycoproteins, and (iii) the nucleic acid composition includes nucleic acid molecules that encode HIV env glycoproteins from primary isolates of at least clades A, B, C, and E.

This claim is designed to cover the vaccine composition designated "DP6-001" in the present application (see, e.g., page 6, last paragraph, and Examples 13 to 16 (as well as others). As will be described in further detail below, this vaccine composition is the one that applicants used in a Phase I human clinical trial, as reviewed in Wang et al. (Exhibit A). Each of these elements distinguish the cited prior art, as will be addressed in further detail below.

The Office alleges that because all of the parts of the claimed invention were known, and the general methodologies were known, it would have been obvious to optimize specific combinations of multiple HIV strains using no more than routine laboratory practice. However, as the public record reflects, it has been extraordinarily difficult to obtain a **protective immune response against HIV in humans**, and applicants' claimed methods have provided the first successful effort at inducing such a protective immune response, in the form of neutralizing

antibodies against multiple primary HIV-1 Env antigens, in humans. No one else has been able to demonstrate such a result in humans prior to applicants' filing of their application.

These results are clearly unexpected in light of the long, yet previously unsuccessful, efforts of others on the road to achieving an effective HIV vaccine, and it is just these types of results that even the U.S. Supreme Court still recognizes as effective evidence to rebut an obviousness rejection (a showing that the elements of an invention work together "in an unexpected and fruitful manner" can support a conclusion that the invention is not obvious. *KSR International Co. v. Teleflex Inc.,* 550 U.S. 398 (2007), slip op. at p. 12).

All of the remaining claims depend from claim 54, including new claim 123, which recites that the sets of nucleic acid molecules encode wild-type HIV envelope gp120 glycoproteins from primary isolates A, B715, Ba-L, Czm, and E, and that the wild-type HIV gag protein is from primary isolate Czm. This claim is designed to specifically cover the vaccine composition designated "DP6-001" in the present application (see, e.g., page 6, last paragraph, and Examples 13 to 16 (as well as others). As will be described in further detail below, this vaccine composition is the one that applicants used in a Phase I human clinical trial, as reviewed in Wang et al. (Exhibit A).

Applicants will now review the meaning of a "protective immune response," as this term is described in the application and in amended claim 54. A protective immune response is one that provides protection against a current or future infection with HIV. Thus, a protective immune response is more than just any immune response. The application describes that a protective immune response can be achieved by neutralizing antibodies generated in the vaccinated host and/or a cell-mediated immune response. These neutralizing antibodies inhibit the HIV virus from replicating and/or proliferating in the vaccinated host. Thus, the key requirement is that the immune response protects the vaccinated individual against a current or future HIV infection (see, e.g., page 6, first paragraph). Many researchers have been able to generate an immune response, e.g., raising antibodies, by vaccination with a DNA-based vaccine, but few have raised an "neutralizing" antibodies in animal models, and no one prior to

applicants has been able to raise such neutralizing antibodies that inhibit the HIV viruses from replicating and/or proliferating in human subjects.

Applicants will next review the cited prior art and the obviousness rejection. First, we will discuss Barnett, the primary reference. Barnett describes administering a DNA prime with **one** gp120 gene and a protein boost with one gp120 protein, which may or may not be the same protein encoded by the gene. While Barnett describes the use of DNA encoding gp120 proteins from clades B and E, these were administered separately to guinea pigs. Barnett does not suggest the use of a multi- or poly-valent composition. Thus, Barnett provides no information itself that would have led skilled practitioners to use combinations of multiple genes and proteins in polyvalent nucleic acid and protein compositions, much less the specific combination of at least four clades A, B, C, and E as presently claimed herein. Barnett also fails to describe any successful efforts at obtaining a protective immune response, e.g., in the form of neutralizing antibodies in humans. While Barnett does describe certain neutralizing antibodies in the guinea pig model, there are no human clinical trials noted.

As Barnett is silent on using polyvalent nucleic acid and protein compositions, this reference does not provide any reasons for skilled practitioners to expect that the recited compositions would work successfully to elicit a broad immune response against a variety of HIV isolates, as further discussed below.

To make up for this deficiency in Barnett, the Office cites Gao 1 and Nabel as describing the concept of polyvalent vaccines, to combine with Barnett. The Office appears to contend that, because Barnett describes using a DNA prime and a protein boost for inducing an immune response against HIV, and that Gao 1 and Nabel disclose various different clades of envelope genes, it would have been obvious to arrive at a method using a polyvalent HIV DNA vaccine and a polyvalent protein boost to induce an immune response against HIV. However, applicants submit that none of the references, individually or combined, suggest using the specific combination of DNA and protein vaccines recited in the presently amended claim 54. Furthermore, the references do not suggest that applicants' claimed methods could successfully induce a protective immune response in humans against a broad spectrum of HIV isolates. Thus,

even assuming that skilled practitioners would have been led to combine the teachings of these references, the instant claims would not have been obvious, as there would have been no reasonable expectation of success.

While Gao 1 describes the cloning and analysis of envelope genes from different HIV-1 clades, this reference does not disclose methods of administering nucleic acid and protein compositions for eliciting immune responses. Gao 1 also does not disclose combinations of genes to be used in such methods. In fact, Gao 1 cautions that "even for subtype B viruses [which had been most widely studied], *the correlates of protective immunity remain unknown.* In the absence of these data, it is *impossible to predict* which combinations of viral antigens from which subtypes are likely to produce the broadest immunity" (Gao 1, page 1651, left col., last line, to right col., lines 2-4, *emphasis added*). Gao 1 also reports technical challenges in producing constructs capable of expressing envelope genes efficiently. Gao 1 states that efficient expression of the env gene for vaccine applications often requires HIV tat and rev proteins. Isolation of cassettes containing these elements "is possible in principle. However...this approach *seems impractical*, at least for large numbers of HIV-1 isolates" (Gao 1, page 1662, left column, fourth full paragraph, to page 1663, left column, first paragraph, *emphasis added*).

Thus, not only does Gao 1 fail to suggest a method of using the specific polyvalent compositions recited in the claims, Gao 1 further suggests that it is not at all obvious which combinations of HIV genes would be useful to provide protective immunity, how many different genes could be included in a polyvalent composition, or whether using a polyvalent composition would even work. Thus, skilled practitioners would not have been led to applicants' claimed methods, or expected that the presently claimed methods would successfully induce broad immune responses against HIV.

These facts are clearly relevant to a proper analysis of obviousness in this case, and it is improper for the Office to cite only the abstract of Gao 1 and simply ignore the contrary information in Gao 1.

Barnett was published in 1997 and Gao 1 was published in 1996, yet it took until applicants' work that culminated in their submitting a paper to the journal *Vaccine*

in September 2007 (and published in 2008, Exhibit A) for anyone to publish on the successful production of neutralizing antibodies against multiple HIV clades in humans.

The Office also cited Nabel as disclosing a method of inducing an immune response against HIV in a mammal using sets of DNA encoded HIV envelope proteins of clade A, C, and E and a panel of DNA encoded HIV gag, and seeks to combine this reference with Barnett and Cao 1. Nabel's earliest filing date for his application is in 2000, yet he fails to even mention using a protein boost anywhere in his lengthy application. Instead, Nabel suggests using a DNA plasmid boost. More importantly, Nabel's invention lies in the use of genetically modified nucleic acid molecules, rather than wild-type nucleic acids, that encode modified HIV glycoproteins. In particular, Nabel deleted cleavage sites and fusogenic domains to expose the core of the Env protein for optimal antigen presentation and recognition (see, e.g., pages 3-4). Nabel also describes a Gag-Pol and Gag-Pol-Nef fusion polypeptide, but does not appear to experiment with wild-type sequences. The goal was to optimize the presentation of epitopes that generate broad CTL and antibody responses. For example, Nabel states, "to improve the immune response to native gp160 and to expose the core protein for optimal antigen presentation and recognition, we have analyzed the immune response to modified forms of the protein" (page 36, lines 14-16).

It is important to get a more complete understanding of the prior art to note that Nabel's group has performed human clinical studies of the very constructs described in the Nabel PCT application. The results of these clinical studies are reviewed in Catanzaro et al., *J. Infectious Dis.*, 194:1638-1649 (2006)(a copy of which is attached as Exhibit B). They immunized human volunteers with a polyvalent vaccine including recombinant adenovirus vectors that encode for an HIV-1 subtype B Gag-Pol fusion protein, and Env glycoproteins from clades A, B, and C. Each of these encoding sequences was modified in some way, e.g., by deletions, replacements, or truncations compared to the wild-type sequences. See the legend of FIG.1 for the details of these modifications. The bottom line result of these trials was that "[n]o neutralizing antibody was detected" (see, Abstract and p. 1644, right column, first full para.). Thus, the modified nucleic

acid based vaccines that are described in the Nabel PCT have not been shown to work to produce a protective immune response in humans.

On the other hand, applicants have been successful in inducing neutralizing antibodies against multiple HIV subtypes in human volunteers. As detailed in Exhibit A, applicants tested a DNA vaccine composition referred to as DP6-001, which as also described in the present patent application, includes six DNA plasmids that encode gp120 glycoproteins from each of the following primary isolates: subtype A, subtype B (92US715.6 and Bal), subtype C (96ZM651), and subtype E, and a sixth plasmid encoding a gag protein, also from subtype C. This DNA vaccine composition was used to immunize healthy adult volunteers, and was followed by a protein boost composition including equal amounts of the five gp120 proteins matching those used in the DNA vaccine (see Materials and Methods, p. 3948).

The results were remarkable, especially in view of the past failures or others. "Positive neutralizing activities against MN were seen in 100% of Groups A and B vaccines sera at the peak antibody level (after the second protein boost)" (page 3951, right column, line 4, to page 3952, left column, line 1). "High-titer NAb [neutralizing antibody] responses (up to 1:2147) were identified in all human immune sera against the three sensitive viruses [MN, NL4-3, and SF162] (Fig. 3b). Using a NAb titer of 1:20 as the cut-off, more than 50% of vaccines also showed positive neutralizing activities against eight pseudotyped viruses [of subtypes A, B, C, D, and E], while the three remaining pseudotyped viruses ... were more resistant to neutralization (Fig. 3c)" (p. 3952, left col., last two lines, to right col., line 8).

Based on these results, the group concluded that "data from this study provides evidence that DNA vaccination can effectively prime the induction of high-level anti-Env antibody responses in humans" (page 3953, right column, lines 2-4). "The DP6-001 formulation elicited neutralizing activities against the sensitive viruses (TCLA and SF162) that were comparable to or better than those elicited by recombinant gp120 alone [30], and clearly much better than a recently reported DNA vaccine alone approach which did not show neutralizing antibody activities [14]. In the current studies, neutralization activity, against pseudoviruses expressing the homologous or randomly selected primary Env antigens, was detected in most of the post-

immunization sera against approximately half of the viruses tested, independent of subtype" (page 3954, left column, line 16, to right column, line 9). While not all Env viruses showed high sensitivity to neutralization, this was "not surprising," and the overall results in this study provide clear evidence of the effectiveness of applicants' claimed invention in comparison of failed prior art efforts.

Applicants submit that the evidence clearly shows that there has been a long-term effort to develop an effective HIV vaccine that provides a protective immune response, i.e., one that induces neutralizing antibodies. Other than applicants' recent publication, applicants are aware of no other group having succeeded in generating such neutralizing antibodies in human subjects. Although others have suggested various approaches, such as Barnett's DNA prime-protein boost approach and Nabel's multivalent modified DNA approach, and although others such as Gao 1 have described the presence of multiple different clades or subtypes of HIV, no one else has been able to achieve what applicants have claimed and then demonstrated in Phase 1 human clinical trials – the induction of neutralizing cross-subtype antibodies from a DNA prime-protein boost HIV-1 vaccine composition.

Based on these surprising results in human clinical trials and the evidence that such results were not at all predictable, much less reasonably predictable, at the time of applicants' claimed invention (and even today), applicants respectfully submit that claim 54 is patentable, and request that the Examiner reconsider and withdraw this rejection. As the Examiner has indicated in the present Action, "[e]vidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness." *In re Rinehart*, 531 F2d 1048, 189 USPQ 143 (CCPA 1976). Applicants have now provided this evidence.

As for the remaining dependent claims, applicants submit that they are all patentable for at least the same reasons. None of Gao 3, Yoshida, or Evans shed any light on the issues addressed above, and thus do not render claim 54 obvious. As a result, applicants' dependent claims to codon optimized sequences, the specific isolates such as Czm and B-al, and the adjuvant QS-21, are patentable for at least the same reasons that claim 54 is patentable. Thus, applicants request that the Examiner reconsider and withdraw all obviousness rejections.

CONCLUSION

Applicants respectfully request that all pending claims be allowed. Applicants do not concede any positions of the Examiner that are not discussed above, nor do applicants concede that there are not other good reasons for patentability of the presented claims or other claims. The extension fee for three months in the amount of $555.00 is being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. Please apply any other charges or credits any other charges or credits to deposit account 06-1050, referencing Attorney Docket No. 7917-0269001.

Respectfully submitted,

Date: April 30, 2009

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EXHIBIT A

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Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime–protein boost HIV-1 vaccine in healthy human volunteers

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ABSTRACT

An optimally effective AIDS vaccine would likely require the induction of both neutralizing antibody and cell-mediated immune responses, which has proven difficult to obtain in previous clinical trials. Here we report on the induction of human immunodeficiency virus type-1 (HIV-1)-specific immune responses in healthy adult volunteers that received the multi-gene, polyvalent, DNA prime–protein boost HIV-1 vaccine formulation, DP6-001, in a Phase I clinical trial. Robust cross-subtype HIV-1-specific T cell responses were detected in IFN-γ ELISPOT assays. Furthermore, we detected high titer serum antibody responses that recognized a wide range of primary HIV-1 Env antigens and also neutralized pseudotyped viruses that express the primary Env antigens from multiple HIV-1 subtypes. These findings demonstrate that the DNA prime–protein boost approach is an effective immunization method to elicit both humoral and cell-mediated immune responses in humans, and that a polyvalent Env formulation could generate broad immune responses against HIV-1 viruses with diverse genetic backgrounds.

1. Introduction

Development of an effective HIV vaccine is critical to control the worldwide AIDS pandemic, which has caused 25 million deaths in the last 25 years and is the cause for more than 40 million people living with HIV/AIDS today [1]. Early efforts in HIV vaccine development focused on the induction of humoral responses by using recombinant Env glycoproteins [2–5]. The immunogenicity of recombinant Env protein-based vaccines was poor in humans, as shown by overall low-level binding antibodies measured by solid phase assays [6] and by the narrow spectrum of neutralizing activities mainly against T cell line adapted (TCLA) viral isolates [7–9]. Ultimately, recombinant protein-based HIV-1 vaccines failed to show protection efficacy in Phase III clinical trials [10,11]. In contrast, recent progress with gene-based vaccination approaches, which have used either DNA or viral vectors as delivery systems, have been effective in eliciting cell-mediated immune (CMI) responses in early phase human studies. However, these studies either did not put forth an effort to elicit protective antibody responses [12,13] or were not effective, when used alone, in eliciting neutralizing antibodies (NAbs) against even relatively sensitive viral isolates [14,15].

Recently, we demonstrated that a DNA prime–protein boost immunization strategy was effective in eliciting humoral and CMI responses in both small animals and non-human primates, including sterilizing immunity in a non-pathogenic SHIV model [16–18]. Our preclinical study results also indicated that this combination vaccination approach, but not recombinant protein alone, was effective in eliciting NAbs against primary HIV isolates [19], a finding that has since been confirmed by other independent studies [20–26]. Furthermore, when polyvalent primary Env antigen formulations were used, the DNA prime–protein boost approach was more effective than the monovalent primary Env antigen in eliciting rabbit NAbs against a wide range of selected primary viral isolates

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across subtypes A–E [27]. In the current study, a multi-gene, polyvalent DNA prime–protein boost HIV-1 vaccine was formulated based on the above preclinical study findings, and its immunogenicity was tested in healthy adults in a Phase I clinical trial. These results demonstrate that this formulation was able to induce balanced cell-mediated and antibody immune responses against HIV-1 antigens, including low but positive neutralizing activities against selected primary HIV-1 isolates across different subtypes.

2. Materials and methods

2.1. Multi-gene, polyvalent, DNA prime–protein boost formulation DP6-001

2.1.1. DNA vaccines

The DP6-001 vaccine contains equal amounts of six individual DNA plasmid components utilizing the same vector pSW3891 [17]: five plasmids each encoding a codon-optimized gp120 gene sequence from the following primary HIV-1 envelope proteins: subtype A (92UG037.8), subtype B (92US715.6 and Bal), subtype C (96ZM651) and subtype E (93TH976.17) and the sixth plasmid encoding a codon-optimized *gag* gene from subtype C (96ZM651) as previously described [28]. The cGMP plasmid DNA for this Phase I clinical trial was produced by Althea Technology (San Diego, CA). The six DNA plasmids used in the DP6-001 vaccine formulation were supplied in saline at a final concentration of 3 mg/ml (0.5 mg/(ml each DNA plasmid)).

2.1.2. Protein vaccines

The recombinant Env protein vaccine components included in the DP6-001 formulation contain equal amounts of five gp120 proteins matching those used in DNA prime components and were produced in CHO cell lines by Advanced BioScience Laboratories (ABL, Kensington, MD) using GMP compliance as previously described [28]. The final protein vaccine product was supplied in saline and re-formulated at the time of injection with 50 μg of QS-21 adjuvant (Antigenics Inc., Woburn, MA) and 30 mg of excipient cyclodextrin (Cargill Cerestar USA Inc., Hammond, IN), to reach a final concentration of 0.375 mg/ml of five gp120 proteins (0.075 mg/(ml each protein)) [28].

2.2. Plasma, sera and antibodies

The HIV human hyperimmune immunoglobulin (HIVIG), HIV-positive patient plasma 91BU003 (infected with a clade C virus) and 93BR029 (infected with a clade B virus) were received from NIH AIDS Research & Reference Reagent Program. Pooled HIV-1 patient sera (infected with clade B viruses) were received from Center for AIDS Researches at UMass Medical School. Normal human sera were purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Phase I clinical study design and sample collection

2.3.1. Participants

Healthy HIV-1-negative adult volunteers aged 18–50 years of both genders were screened. The individuals enrolled in this Phase I trial had no history of chronic, allergic and immunodeficient illnesses, organ transplantations or psychiatric disorder, were negative in hepatitis B and C viral tests and a pregnancy test for all female subjects was negative. All subjects were recruited at the single clinical trial site at the University of Massachusetts Medical School (UMMS), Worcester, MA, according to IRB approved study protocol.

2.3.2. Study design and immunization schedule

This open-label Phase I trial involved two dose levels of DNA prime and a single dose level of protein boost. The study design and numbers of volunteers included in the current analysis are provided in Table 1. Volunteers were randomly assigned to either Group A or B (1.2 mg of DNA at each immunization) at first and enrollment to Group C was started only after the safety review on Groups A and B volunteers who received the second protein boost. DNA vaccine was administrated by intradermal (ID) injection at four sites (0.3 mg in 0.1 ml per site) in Group A and by intramuscular (IM) injection at two sites (0.6 mg in 0.2 ml per site) in Group B. Group C received a sixfold higher dose of the DNA vaccine (7.2 mg at each immunization) via IM injection at two sites (3.6 mg in 1.2 ml per site). Each volunteer received three priming vaccinations of DNA vaccines at study weeks 0, 4 and 12 and two booster immunizations of protein vaccinations via single site IM injection at study weeks 20 and 28 (Table 1). The adjuvant, QS-21, and excipient, cyclodextrin, were mixed with the five gp120 proteins in a total volume of 1 ml at the time of injection.

Serum and PBMC samples were collected at study weeks 0, 2, 4, 6, 12, 14, 16, 20, 22, 24, 28, 30, 32, 36 and 52 to measure antibody and CMI responses. All volunteers were recruited and enrolled in the Clinical Vaccine Research Unit, UMMS. For the current immunogenicity report, samples from the following 27 volunteers are included: Group A ($n=10$) and Group B ($n=11$) volunteers who completed the entire three DNA and two protein immunizations and Group C volunteers ($n=6$) who received the three DNA and one protein immunization (Table 1).

2.4. ELISPOT

The interferon (IFN)-γ ELISPOT assay for HIV-1 peptide-specific T cells used the human IFN-γ ELISPOT kit from Mabtech (Cat# 3420-2) (Cincinnati, OH) with Millipore (Billerica, MA) ELISPOT plates, MSIPS4W10. The assay was performed according to manufacturers' directions with the minor modifications that monoclonal antibody, 1-D1K, was diluted to 5 μg/ml and biotinylated monoclonal antibody, 7-B6-1, was diluted to 2 μg/ml. The ELISPOT assay was performed using pools of peptides covering gp120 of HIV-1 92UG037.8 (Env-A), 92US715.6 (Env-B), 96ZM651 (Env-C) and 93TH976.17 (Env-E) or Gag of 96ZM651 that are present in the vaccine formulation. These peptides, 20-mer in length and overlapping by 10 amino acids, were produced in the UMMS Peptide Core Facility. Each peptide pool consisted of five to seven peptides with a final concentration of 2 μg/ml per peptide. Plates were developed using the Vector Nova Red Kit (SK-4800, Vector Labs, Burlingame, CA).

Cryopreserved PBMCs were thawed, washed and diluted to 2×10^5 per well in RPMI 10 (10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin). Wells containing media alone served as a negative control and wells containing 1 μg/ml phytohemagglutinin (PHA) served as a positive control. A CEF peptide pool containing defined epitopes of cytomegalovirus, Epstein-Barr virus and influenza A virus was used as a peptide positive control [29]. PBMC from HIV-1-infected donors and healthy control donors (with a known positive response to the CEF peptide pool) were included in each assay to assess reproducibility. Selected samples were repeated to test inter-assay variation. The final IFN-γ ELISPOT responses against Env or Gag peptides of each individual antigen were calculated as the cumulative response across non-overlapping peptide pools. Spots were counted and analyzed on a CTL immunospot 3 reader and recorded as the mean spot-forming cells (SFC) per million PBMCs of replicate or triplicate wells. The final numbers of

Table 1
Study design of DP6-001 clinical trial

Group	DNA prime phase				Protein boost phase			
	Plasmids (6)	Dose (mg)	Route	Time (weeks)	Proteins (5)	Dose (mg)	Route	Time (weeks)
A (n = 10)	gp120 (A, B, Bal, C, E) + Gag (C)	1.2	ID	0, 4, 12	gp120 (A, B, Bal, C, E)	0.375	IM	20, 28
B (n = 11)	gp120 (A, B, Bal, C, E) + Gag (C)	1.2	IM	0, 4, 12	gp120 (A, B, Bal, C, E)	0.375	IM	20, 28
C (n = 6)	gp120 (A, B, Bal, C, E) + Gag (C)	7.2	IM	0, 4, 12	gp120 (A, B, Bal, C, E)	0.375	IM	20

HIV-1 viral strains (GeneBank accession number) used for DNA or protein vaccines. (A) 92UG037.8 (U09127), (B) 92US715.6 (U08451), Bal: Ba-L (M68893), (C) 96ZM651 (AF286224), (E) 93TH976.17 (U08458).

peptide-specific SFC were obtained by subtracting the background spots in medium control wells. The cut-off for positive responses was determined as at least 10 peptide-specific SFC per million PBMCs.

2.5. ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to detect the gp120-specific IgG responses. The gp120 antigens used in the ELISA assays were five proportionally pre-mixed gp120 antigens included in the DP6-001 formulation produced in CHO cells with a purity of >99% based on size exclusion HPLC. Microtiter plates were coated with the five-mixed gp120 antigens at 100 ng/well (20 ng/antigen) in PBS (100 μl) at 4 °C for 1 h; plates were then washed and blocked in 200 μl blocking buffer (PBS, 0.5% Tween-20, 5% NGS, 5% non-fat dry milk) overnight at 4 °C. On the following day, serum dilutions were prepared in Dilsim II (BioMerieux, Durham, NC) and incubated on the plates (100 μl/well) for 1 h at room temperature. Biotinylated goat anti-human IgG (Vector Laboratories, CA) was diluted to 1:5000 in Dilsim II and then incubated on the plates (100 μl/well) for 1 h at room temperature. Horseradish peroxidase–streptavidin (Vector Laboratories, CA) diluted to 1:10,000 in Dilsim II was added (100 μl/well) to the plates and incubated for 1 h at room temperature. Between steps, the plates were washed five times with 1× PBS–0.1% Triton X-100. The assays were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO) and stopped with sulfuric acid after 3 min. Assays were read immediately at 450 and 630 nm using the Opsys MR Microplate Reader (Dynex Technologies, Chantilly, VA). The gp120-specific antibody titer was determined as the highest serum dilution which achieved twofold higher OD value than the pre-bleed control for each subject.

2.6. Western blot

The individual gp120 antigens used in the Western blot analysis were obtained from different sources: A, B, Bal, C, E and 92BR025.9 from ABL as the same reagents used for ELISA; JR-FL, ADA, SF162, CN54 and CM235 from NIH AIDS Reagent Program; CA1 and UG21-9 from NIH/NIAID; 92UG021, 93BR020.17 and 92UG975.10 produced from 293T cells at UMMS. Individual gp120 antigens (100 ng/lane) were subjected to SDS–PAGE and blotted onto PVDF membrane, as previously described [27]. Blocking of PVDF membrane was done with 0.1% I-Block (Tropix, Bedford, MA). The membranes were incubated with sera at 1:100 dilution for 45 min and subsequently reacted with AP-conjugated goat anti-human IgG (Tropix, Bedford, MA) at 1:5000 dilution for 30 min. Membranes were washed with blocking buffer after each step. Western-light substrate was then applied to the membranes for 5 min. Once the membranes were dried, X-ray films were exposed to the membrane and developed by a Kodak processor. Individual HIV+ patient sera or purified HIV+ immunoglobulin (HIV-IG) were used as controls.

2.7. Neutralizing antibody assays

One virus neutralization assay was based on reductions in luciferase (Luc) reporter gene expression after a single round of virus infection with pseudotyped HIV-1 viruses in TZM-bl cells, as previously described [30]. Neutralizing antibody levels in the human sera from DP6-001 vaccines and normal control donors were measured against two panels of pseudotyped HIV-1 viruses expressing primary Envs antigens. There were six pseudotyped HIV-1 viruses in the first panel (Tier 1) including MN and five homologous to the Env immunogens included in the DP6-001 formulation. There were 12 viruses in the second panel (Tier 2), 4 each from subtype B (QH0692042, SC422661.8, PVO.4 and AC10.0.29), subtype A (Q23.17, Q168.A2, Q461.E2 and Q769.D22), and subtype C (Du123.6, Du151.2, Du156.12 and Du172.17) [31,32]. In this assay, 200 $TCID_{50}$ of virus was incubated with diluted serum samples in triplicate in a total volume of 150 μl for 1 h at 37 °C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 μl of growth medium containing 75 μg/ml DEAE dextran) were added to each well. One set of control wells received cells plus virus (virus control) and another set received cells only (background control). After 48 h incubation, 100 μl of cells was transferred to a 96-well black solid plate (Costar) for measurements of luminescence using Bright Glo substrate solution, as described by the supplier (Promega). The percent neutralization was calculated by comparing experimental wells to virus control wells. Neutralization titer was the dilution at which RLUs were reduced by 50% compared to virus control wells after subtraction of background RLUs using pre-bleed sera.

The second neutralization assay (PhenoSense™ assay) used recombinant viruses pseudotyped with the virus envelope proteins and a firefly luciferase indicator gene [33]. The pseudoviruses were incubated for 18 h at 37 °C with serial threefold dilutions of heat-inactivated human sera. U87 cells that express CD4 plus the CCR5 and CXCR4 co-receptors were inoculated with virus dilutions in the absence of added cations. Virus infectivity was determined 3 days later by measuring the amount of luciferase activity expressed in infected cells. Neutralizing activity was calculated as the percent inhibition of viral replication (luciferase activity) at each antibody dilution compared with an antibody-negative control: % inhibition = {1 − [luciferase + Ab/(luciferase − Ab)]} × 100. Titers were presented as the reciprocal of the plasma dilution conferring 50% inhibition (IC50) [33]. The specificity control was composed of a virus pseudotyped with an aMuLV envelope. An HIV-serum combination was considered to have positive neutralization if the inhibition of HIV was at least 50% and >3× higher IC50 than the same plasmas tested with aMuLV while the pre-bleed was not scored positive. The starting sera dilution used in the neutralization assays was 1:20.

2.8. Statistical analysis

Wilcoxon rank sum test was used to analyze the differences of HIV-1 antigen-specific T cell ELISPOT and antibody responses

between vaccination groups. Student's *t*-test was used to analyze the difference of T cell ELISPOT results between low- and high-dose DNA primed groups (Group A/B vs. Group C).

3. Results

3.1. Design of the Phase I clinical trial in healthy adult volunteers

The multi-gene, polyvalent primary Env DNA prime–protein boost HIV vaccine, DP6-001, included six DNA plasmids (one expressing a subtype C full length Gag antigen and the other five each expressing one of the five primary gp120 antigens from subtypes A, B, C or E) as the prime and five recombinant gp120 proteins matching the Env DNA prime as the boost (Table 1). The study was a three-group trial that tested two dosing levels of DNA administered either intradermally (ID) or intramuscularly (IM) and one standard dose of protein with adjuvant QS-21 administered IM (Table 1). Groups A and B received three DNA immunizations, either ID or IM, respectively, with 1.2 mg total DNA at each immunization, equally divided between one Gag and five gp120 DNA plasmids. For Group C, a higher dose of DNA prime with 7.2 mg total DNA was administered IM at each immunization. Protein boosts contained a fixed dose of five recombinant gp120 proteins delivered twice with adjuvant QS-21.

Overall the DP6-001 vaccine was well tolerated and the most frequent adverse events were skin reactions. A higher reactogenicity, in the form of transient low grade fever and one case of lower extremity leukocytoclastic vasculitis (LCV), a skin form vasculitis with unknown etiology, which was resolved shortly without specific treatment, was observed in the high-dose DNA prime group (Group C) after receiving a protein boost. Due to such reactogenicity, the clinical trial was terminated early and only six subjects in Group C received one protein boost. Detailed information on the safety and reactogenicity of this clinical trial is summarized in a separate report (Kennedy et al., submitted for publication). The current report focuses on the immunogenicity results in volunteers received DP6-001.

3.2. Cross-subtype HIV-1-specific cell-mediated immune responses

CMI responses to two primary Env (Env-A and Env-B) antigens and one Gag antigen were first analyzed by an IFN-γ ELISPOT assay. Positive CMI responses against pooled Env peptides were observed at the end of the three DNA prime immunizations for both low-dose DNA priming groups (Groups A and B) but the levels of CMI responses were low (Fig. 1a). The high-dose DNA prime (Group C) was able to induce a significantly higher CMI response at the end of the DNA priming phase when compared to Groups A and B ($p < 0.01$ for CMI responses against both Env-A and Env-B antigens).

Levels of Env-specific CMI were further boosted by each of the two subsequent protein immunizations and maintained at relatively high levels even at the end of the trial (24 weeks from the last protein immunization). Higher CMI responses to Env-A and Env-B antigens were observed in Group C volunteers when compared to Group A ($p < 0.05$) and Group B ($p < 0.05$), showing the response of a sixfold higher dose of the DNA vaccine during the priming immunizations (Fig. 1a).

Also, while CMI responses to Gag peptides were poor in Groups A and B, these responses increased substantially in Group C ($p < 0.05$ compared with either Group A or B) (Fig. 1a and Table 2). The Gag-specific CMI response declined more than fivefold from its peak level at the end of DNA prime immunization to barely positive responses by 52 weeks while the Env-specific CMI declined only two- to threefold from peak levels (Table 2). It is not clear whether such a difference was due to the fact that only an Env protein was used as the boost.

Overall, 90–100% of the volunteers had positive Env-specific IFN-γ ELISPOT responses against both Env-A and Env-B peptides after two protein boosts in low-dose Groups A and B, and 100% had positive responses by the end of DNA priming in high-dose Group C (Table 2). At week 52, greater than 80% of the volunteers still had positive Env-specific CMI responses. In contrast, although 100% of volunteers in Group C had positive Gag-specific IFN-γ ELISPOT with DNA prime alone, the percentage of responders decreased to about 50% by week 52 (Table 2).

Additional IFN-γ ELISPOT analyses using peptides from the subtypes C and E Env antigens included in DP6-001 were conducted with samples from six randomly selected volunteers from each group. Env-C- and Env-E-specific CMI responses were detected in all three groups but higher peak levels of positive CMI responses at the end of DNA prime were observed in Group C when compared to Group A or B (Fig. 1b). Flow cytometry assays further demonstrated that the HIV-1-specific IFN-γ responses observed in this study were mediated predominantly by CD4+ T cells. HIV-1-specific CD8+ T cell responses were also detected but at a lower frequency. Detailed flow cytometry results will be summarized in a separate report [49].

3.3. Broadly reactive antibody responses against a wide range of primary HIV-1 Env antigens

High titer HIV-1 Env-specific antibody responses were generated with the DNA prime–protein boost DP6-001 formulation (Fig. 2a). For Groups A and B, most volunteers did not have detectable Env-specific antibody responses at the end of three DNA prime immunizations, as expected at such a low dose of DNA [14,34]. However, the antibody titers rose quickly after just one protein boost. The anti-gp120 IgG titers reached $1{:}10^5$ or higher with one or two protein boosts, levels that are comparable to those observed in chronically infected HIV patients (Fig. 2a). The majority of volunteers maintained significant serum anti-gp120 IgG titers at the end of trial. Four out of six volunteers (66.7%) in Group C showed detectable Env-specific IgG responses even before protein boosting (Fig. 2a and Table 2). The gp120 antigens used in the ELISA assay were the same GMP lot proteins produced from CHO cells used for DP6-001 formulation. The specificity of antibody responses were further confirmed by another ELISA against Env antigens produced from a different cell line 293T cells (data not shown) and Western blot analysis (see below).

Western blot analysis indicated that antibodies elicited by the polyvalent Env formulation, DP6-001, were broadly cross-reactive against a diverse panel of primary HIV-1 Env antigens (Fig. 2b). Sera from all 27 volunteers included in this report recognized all of the five homologous primary gp120 antigens included in DP6-001 after one or two protein boosts, as shown by two representative volunteer sera from Group A (#022) and Group B (#023). Neither randomly selected individual HIV+ patient serum nor pooled HIV-1-positive human immunoglobulin (HIV-IG) from patients infected with subtype B viruses could recognize more than two or three primary gp120 antigens (Fig. 2b). Additional Western blot analysis confirmed reactivity of immunized volunteer sera against a wide range of 11 heterologous primary HIV-1 gp120 antigens from subtypes A to G. One representative sample blot with volunteer #013 sera is shown in Fig. 2c. Two control individual HIV+ patient sera could only recognize a small fraction of these primary gp120 antigens.

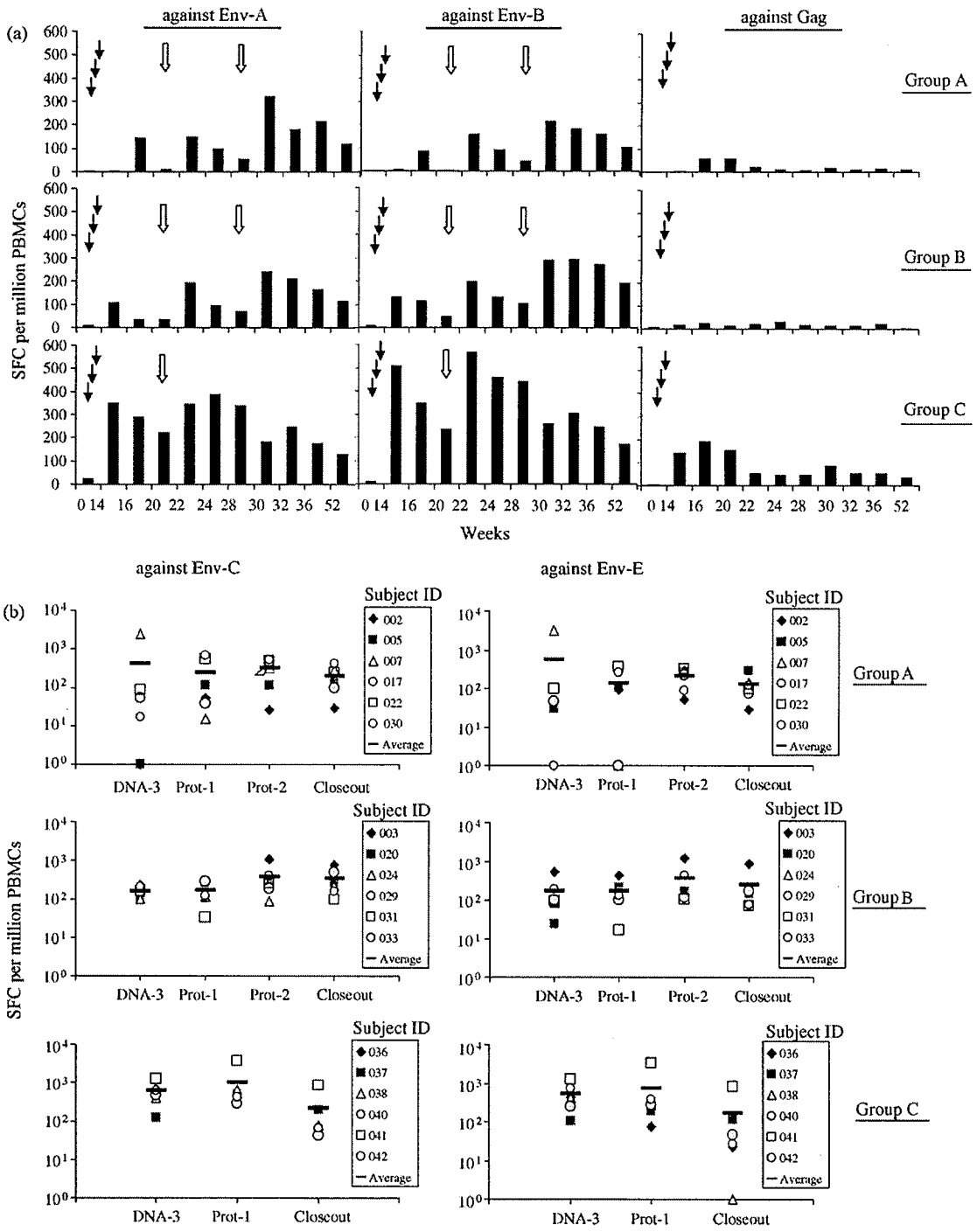

Fig. 1. DP6-001 formulation induced HIV-1-specific cell-mediated immune responses in volunteers' PBMC. (a) Group averages of Env- and Gag-specific IFN-γ ELISPOT responses at different time points following DNA or protein immunizations (see Table 2 for standard error and percentage of responders). The solid arrows indicate DNA immunizations and the open arrows indicate protein immunizations. Pools of overlapping peptides from either gp120 antigens of subtype A isolate 92UG037.8 (Env-A) and subtype B isolate 92US715.6 (Env-B) or Gag antigen of subtype C isolate 96ZM651 (Gag) were used for the assay. (b) HIV-1-specific IFN-γ ELISPOT responses were detected at 2 weeks after either the third DNA immunization (DNA-3), the first or second protein boosts (Prot-1 and Prot-2), or at week 52 (closeout) against pools overlapping peptides from gp120 antigens of subtype C isolate 96ZM651 (Env-C) and subtype E isolate 93TH976.17 (Env-E). Results are shown as responses from each of six randomly selected volunteers in each group as well as the group average (short horizontal bars).

3.4. Neutralizing antibody activities of DP6-001 vaccinated human sera

Three studies were organized to assess the neutralizing antibody activities in DP6-001 vaccinee sera. The first study analyzed the ability of these sera to neutralize the Tier 1 viruses: a TCLA HIV-1 virus (MN) and five pseudotyped viruses each expressing one of the five homologous primary Env antigens included in DP6-001 (Table 3 and Fig. 3a). Positive neutralizing activities against MN were seen in 100% of Groups A and B vaccinee sera at the peak

Table 2
Summary of HIV-1 antigen-specific cell-mediated immune responses and gp120-specific antibody responses in DP6-001 vaccinees

Samples	Peptide-specific IFN-γ ELISPOT (SFC/million PBMCs)[a]						gp120-specific IgG titers[b]		
	Against Env-A		Against Env-B		Against Gag		Median	Range	% Responders
	Average + S.E.	% Responders	Average + S.E.	% Responders	Average + S.E.	% Responders			
Group A (n = 10)									
DNA-3	149.5 ± 112.3	70	83 ± 60.9	50	56 ± 42.3	30	<50	<50–50	20
Protein-1	147.5 ± 70	70	185.1 ± 81.7	80.0	NA	NA	25,600	200–1,638,400	100
Protein-2	322.9 ± 82.3	100	214.1 ± 68.1	90.0	NA	NA	204,800	25,600–819,200	100
Closeout	119.5 ± 25	80	102.2 ± 33.9	80.0	10.1 ± 7.7	20	12,800	<50–51,200	90
Group B (n = 11)									
DNA-3	108.6 ± 27.2	100	131.7 ± 35.1	81.8	25 ± 13.6	36.4	<50	<50	0
Protein-1	195.8 ± 94.3	81.8	201.4 ± 97.7	100	NA	NA	25,600	200–819,200	100
Protein-2	242.4 ± 100.4	100	291.5 ± 132.7	100	NA	NA	204,800	1600–819,200	100
Closeout	151.3 ± 57.1	81.8	192.7 ± 70.3	90	4.3 ± 2.8	18.2	8000	200–102,400	100
Group C (n = 6)									
DNA-3	348.1 ± 122.9	100	507.4 ± 161.4	100	191.5 ± 86.1	100	1600	<50–409,600	66.7
Protein-1	347.5 ± 203.1	100	569.2 ± 394.2	100	NA	NA	51,200	51,200–819,200	100
Protein-2	NA	NA	NA	NA	NA	NA	NA	NA	NA
Closeout	131.4 ± 97.2	83.3	171.0 ± 114.2	83.3	35.6 ± 16.8	50	8000	800–25,600	100

NA: not applicable.

[a] SFC: spot-forming cells were measured against Env-A, Env-B and Gag peptides at 2 weeks after the third DNA (DNA-3), the first and second protein (protein-1 and protein-2) immunizations, and at week 52 (closeout), respectively.

[b] gp120-specific IgG titers were measured against five-mixed gp120 proteins (A, B, Bal, C and E) at 2 weeks after the third DNA (DNA-3), the first and second protein (protein-1 and protein-2) immunizations, and at week 52 (closeout), respectively.

antibody level (after the second protein boost). The percentage of positive NAb responses against different homologous pseudotyped viruses varied: 71% neutralized Bal (subtype B), 62% neutralized 96ZM651 (subtype C), 38% neutralized 93TH976 (subtype E), 28% neutralized 92UG037 (subtype A), and less than 10% neutralized 92US715 (subtype B). None of the seven control human serum samples had positive neutralizing activity against the above viruses (Table 3). More than 50% of the vaccinees had NAb titers greater than 1:100 against MN. The titers of NAbs against other homologous pseudotyped viruses were between 1:20 and 1:100 for vaccinee sera with positive NAb activities (Fig. 3a). Group C showed a higher percentage of individual positive NAb sera after only one protein boost when compared to corresponding sera from Groups A and B. One vaccinee (16.67%) in Group C even had positive NAb activities against MN, Bal and 96ZM651 following only the DNA immunizations (Table 3).

The second neutralization study was done using a high-throughput, pseudotyped virus assay system. Sera collected 2 weeks after the second protein boost (peak of anti-gp120 antibody responses) were assayed against a panel of 11 heterologous primary viruses covering subtypes A–E in addition to three sensitive viruses (MN, NL4-3 and SF162). Because Group C volunteers did not complete two protein boost immunizations, only sera from Groups A and B volunteers are included in this study. High-titer NAb responses (up to 1:2147) were identified in all human immune sera against the three sensitive viruses (Fig. 3b). Using a NAb titer of 1:20 as the cut-off, more than 50% of vaccinees also showed positive neutralizing activities against eight pseudotyped viruses (92UG103 and 92RW020 of subtype A, 92BR020 and 92US715 of subtype B, 98CN006 and 93IN905 of subtype C, 92UG046 of subtype D and 92TH021 of subtype E), while the three remaining pseudotyped viruses (JR-CSF of subtype B, 94UG114 of subtype D and CMU02 of subtype E) were more resistant to neutralization (Fig. 3c). The overall patterns of NAb responses were similar between Groups A and B. Two vaccinees from Group A (#007 and #009) and two from Group B (#010 and #016) had NAb activities against a majority of the pseudotyped viruses included in the analysis.

The third neutralization study was conducted to test the ability of DP6-001 vaccinee sera to neutralize standardized panels of Tier 2 viruses. Viruses included in these panels are more recently isolated (the "contemporary viruses") from acute and early infections [31]. Although neutralizing activity was clearly higher than the negative control sera, most volunteer sera displayed viral inhibition of less than 50% at a serum dilution of 1:10 (Fig. 3d).

4. Discussion

Induction of anti-Env antibody responses in small animals was one of the first pieces of evidence that established DNA immunization as a novel approach for vaccination [35]. Although significant

Table 3
Percent of responders with neutralizing antibody (NAb) titers greater than 1:20 (Study 1)

Serum samples	% with positive NAb against autologous pseudotyped HIV-1 viruses (subtype)					
	MN (B)	Bal (B)	92US715 (B)	92UG037 (A)	96ZM651 (C)	93TH976 (E)
Groups A and B (n = 21)						
DNA-3	0.00	4.76	0.00	4.76	0.00	0.00
Protein-1	28.57	28.57	0.00	4.76	14.29	4.76
Protein-2	100.00	71.43	9.52	28.57	61.90	38.10
Group C (n = 6)						
DNA-3	16.67	16.67	0.00	0.00	16.67	0.00
Protein-1	66.67	50.00	0.00	0.00	33.33	0.00
Protein-2	NA	NA	NA	NA	NA	NA
Negative control (n = 7)	0.00	0.00	0.00	0.00	0.00	0.00

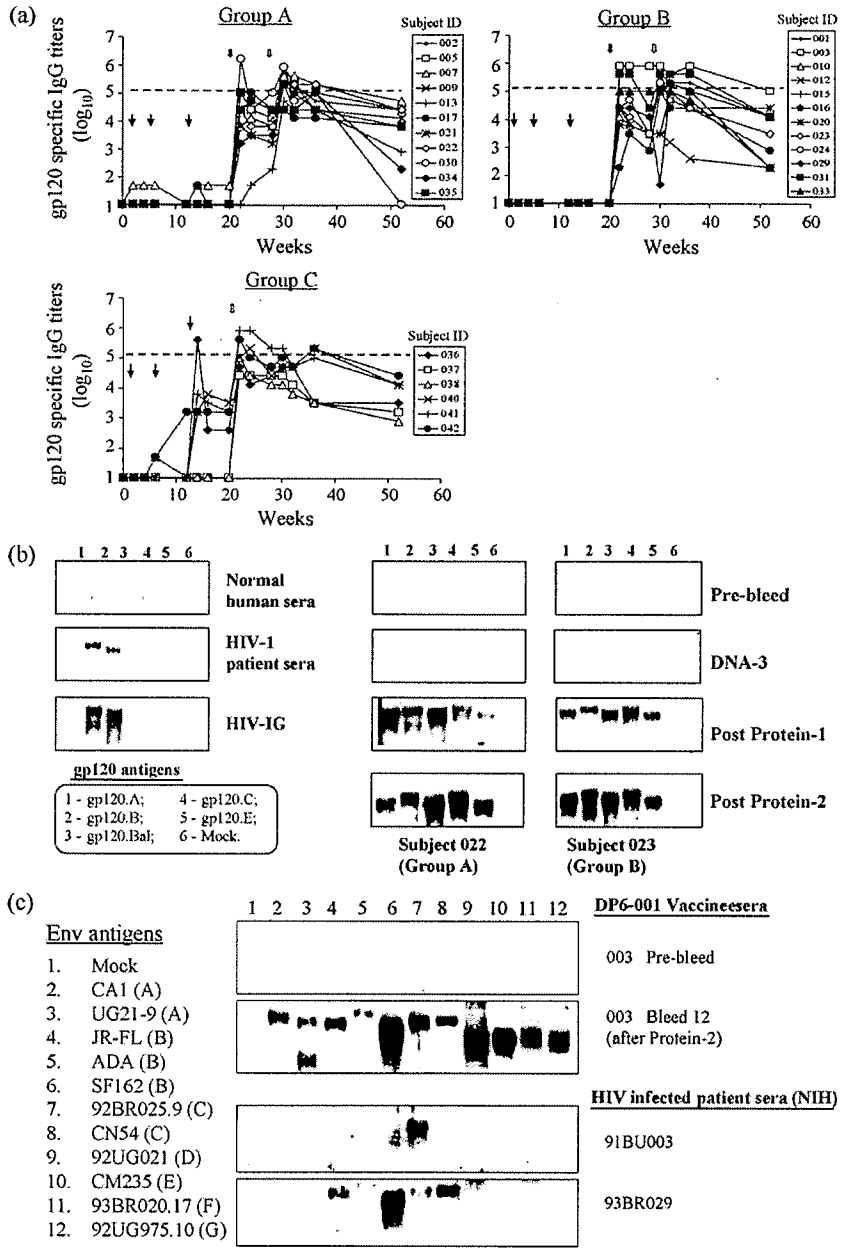

Fig. 2. DP6-001 formulation induced HIV-1 gp120-specific antibody responses in volunteers' sera. (a) Titers of serum gp120-specific IgG were measured by ELISA in individual volunteers of different study groups. Each curve represents one volunteer. The solid arrows indicate DNA immunizations and the open arrows indicate protein immunizations. The dotted line denotes the average titer of gp120-specific IgG in sera of three patients chronically infected with HIV-1 (titer range: 1:102,400 to 1:204,800). (b) Reactivity of two DP6-001 immune sera (#022 of Group A and #023 of Group B at 1:100 dilution) at different time points of immunization against five autologous primary HIV-1 gp120 glycoproteins as measured by Western blot analysis. Normal human sera and HIV-1-positive patient sera, both at 1:100 dilution, or HIV-1-positive immunoglobulin (HIV-IG) (at 0.5 mg/ml total human IgG) were included as controls. (c) Cross-reactivity of sera from one representative DP6-001 immune serum #003 (Group B) against a panel of heterologous primary HIV-1 gp120 glycoproteins by Western blot analysis. Two individual HIV+ patient sera (91BU003 and 93BR029) were included as the controls. Serum dilution of 1:100 was used for both DP6-001 and HIV+ patient sera.

progress has been made using DNA immunization to elicit HIV-1-specific CMI in small animals, non-human primates and humans over the past 15 years [12–16,18,36–39], there has been no report of using Env DNA immunization to elicit broadly cross-reactive antibodies in humans. Levels of anti-Env antibodies elicited in previous DNA vaccine clinical trials were either low or undetectable [40,41], nor there was a clear induction of NAbs against even sensitive viruses [14]. In the current study, one Env protein immunization following DNA prime was able to elicit human anti-Env antibody responses to a level that has proven difficult to achieve in previous studies that employed multiple injections of recombinant HIV-1 Env proteins [2,3,6]. Therefore, data from this study provides evidence that DNA vaccination can effectively prime the induction of high-level anti-Env antibody responses in humans.

In prior recombinant Env protein-based clinical HIV vaccine studies, up to two Env antigens were included in the vaccine formulation and as many as seven Env protein immunizations had to be administered in order to achieve high antibody responses [42]. While the overall levels of Env-specific antibodies were low and not broadly cross-reactive, their binding specificity was measured

against selected peptides of Env proteins rather than the primary Env antigens themselves [4,43]. Our results clearly demonstrate the benefit of delivering polyvalent Env antigens through the DNA prime–protein boost approach. Not only did we elicit Env-specific antibody responses comparable to those seen in persons chronically infected with HIV-1, but the immune sera were able to react with primary Env antigens from every HIV subtype included in the study. Furthermore, the antibody responses appeared to be long lasting, with only a moderate decrease in titers at the end of the 52-week study period.

Given the importance of NAb responses in a prophylactic HIV vaccine and the complexity of measuring such responses, three neutralization studies were conducted, each with a slightly different approach and each using different panels of HIV viruses, to provide a more complete picture of the spectrum of neutralizing activities contained in the immune sera. The DP6-001 formulation elicited neutralizing activities against the sensitive viruses (TCLA and SF162) that were comparable to or better than those elicited by recombinant gp120 alone [30], and clearly much better than a recently reported DNA vaccine alone approach which did not show neutralizing antibody activities [14]. In the current studies, neutralization activity, against pseudoviruses expressing the homologous or randomly selected primary Env antigens, was detected in most of the post-immunization sera against approximately half of the viruses tested, independent of subtype. Not all homologous Env viruses showed high sensitivity to neutralization by post-immunization sera, which was not surprising given previous reports showing that infected HIV patient sera did not always have a high neutralizing activity against autologous viruses [44,45].

The neutralizing activities against the standard panel of Tier 2 HIV-1 viruses were low in the current study, even at low serum dilutions. While it is unclear whether this panel represents more

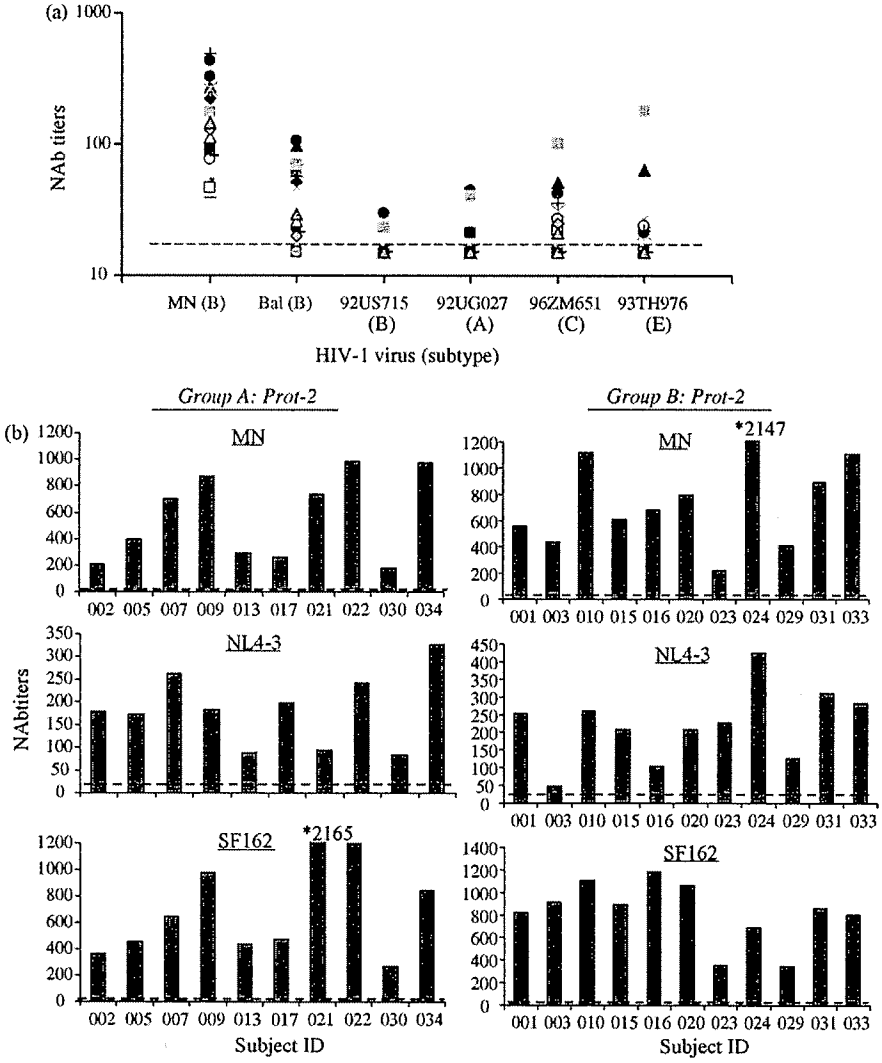

Fig. 3. Neutralizing antibody (NAb) responses against different panels of pseudotyped viruses. (a) NAb titers against Tier 1/autologous viruses including a T-cell line adapted (TCLA) HIV-1 virus (MN) and five pseudotyped viruses each expressing one of the five autologous primary Env antigens included in the DP6-001 formulation. The neutralization assays were done in TZM-bl cells and the NAb titers were measured using individual volunteer sera from Groups A and B ($N = 21$) at 2 weeks after the second protein boost. (b and c) NAb titers determined by PhenoSense™ assay in U87 cells with sera from Groups A and B at 2 weeks after the second protein boost against pseudotyped viruses expressing either (b) three relatively sensitive to neutralization Env antigens of subtype B (MN, NL4-3 and SF162) or (c) 11 additional primary Env antigens of subtypes A–E. A serum dilution of 1:20 was used as the cut-off to score the positive NAb (shown as the broken line in each graph). (d) NAb responses against Tier 2 pseudotyped viruses expressing primary Env from subtypes A–C (four viruses for each subtype). Individual subject sera from Groups A and B were collected at 2 weeks post the second protein boost. The percent neutralization was measured at 1:10 serum dilution. The neutralization assays were done in TZM-bl cells.

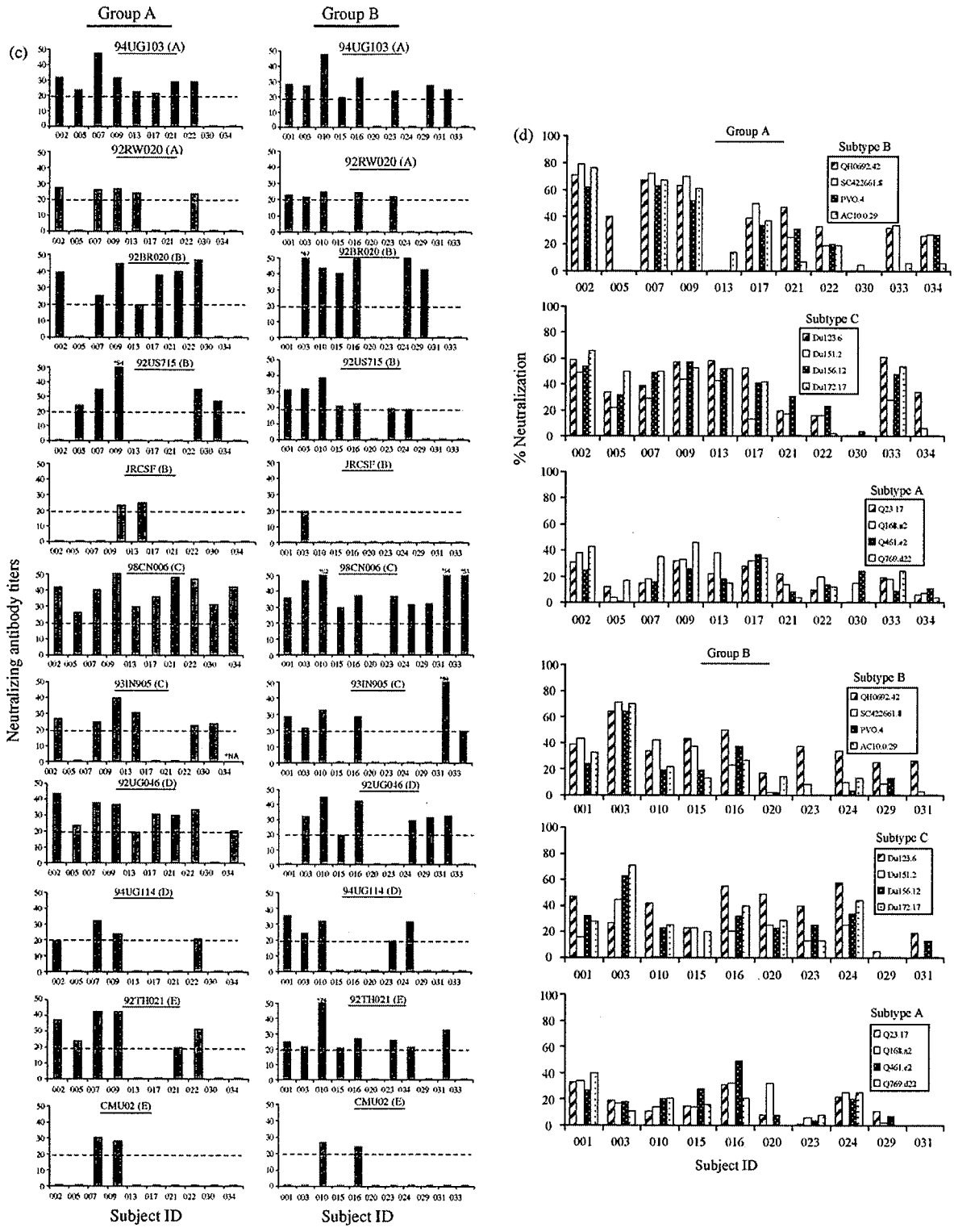

Fig. 3. (*Continued*).

resistant viruses than the viruses included in the other two neutralization assays, it is possible that the difference reflects the inclusion of more contemporary viral isolates in the Tier 2 panel while Env antigens included in the DP6-001 formulation were cloned from patient samples collected around or before the early 1990s. While the current DP6-001 formulation, as a proof of concept study, may not have the optimal profile to move to more advanced clinical trials, the results included in this report clearly indicate that the use of a polyvalent, DNA prime–protein boost approach is feasible in order to elicit human neutralizing activities against a wide range of HIV-1

isolates. Future studies should test whether modified formulations including contemporary Env antigens can improve neutralizing activities against Tier 2 viruses.

Recently published studies with optimized DNA or other gene-based HIV vaccines elicited robust CMI responses [14,15]. One key question asked in the current trial was whether robust CMI responses would be maintained when DNA immunization was used in conjunction with a protein boost component. The levels of Env-specific CMI responses elicited after three DNA immunizations in this study were similar to those previously reported [12,14,15]. One new finding from the current study is that the Env protein boost further increased the magnitude of Env-specific CMI responses when compared to the Gag-specific CMI, for which there was no protein boost. The Env-specific CMI responses were cross-reactive against at least four different primary Env antigens that were included in this study. In further support for the presence of HIV-1-specific CMI responses in DP6-001 vaccinees, a subset of volunteers developed DTH-like skin reactions at the sites of DNA immunization after receiving an Env protein boost at a distant inoculation (Kennedy et al., submitted for publication).

We also detected a robust Gag-specific CMI response in Group C volunteers, which was higher than previously observed by others [14,15]. Given the 1:5 ratio of Gag to Env DNA vaccines in DP6-001, the actual dose of Gag DNA vaccine was 1.2 mg in Group C, suggesting that the minimum immunogenic dose for naked DNA vaccines would be at least 1 mg with the current design of DNA vaccines. There is a clear dose–response relationship for both Env and Gag DNA vaccines. Due to the small sample size in the current study, future trials should study the boost effect of proteins on CMI responses. Furthermore, this study also shows that the ID route of administration was not more effective than IM in eliciting an immune response by DNA vaccines.

In summary, results from this first report of a human study with DNA prime–protein boost HIV-1 vaccine confirms the immunogenicity of this novel approach in eliciting balanced humoral and cell-mediated immune responses in a healthy adult population. These results are significant in that they confirm initial observations on the immunogenicity of DNA vaccines in animal models [35,46–48]. The DNA prime–protein boost approach will not only accelerate the testing of more candidate HIV vaccines that aim to achieve improved neutralizing antibody responses, but will also provide a new platform for the development of future vaccines against a wide range of existing or emerging pathogens. Future studies should include in-depth analysis on the structural basis for both antibody and CMI cross-reactivities observed in the current report. The composition of Env antigens should be further optimized to identify a polyvalent formulation that may expand the breadth of neutralizing activities against viruses that were resistant to immune sera elicited by DP6-001. The immunization schedule including the use of adjuvant should be also optimized to reduce the reactogenicity of DP6-001 before moving this DNA prime and protein boost approach to more advanced human studies.

Acknowledgements

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EXHIBIT B

MAJOR ARTICLE

Phase 1 Safety and Immunogenicity Evaluation of a Multiclade HIV-1 Candidate Vaccine Delivered by a Replication-Defective Recombinant Adenovirus Vector

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(See the editorial commentary by Robinson and Weinhold and the article by Graham et al., on pages 1625–7 and 1650–60, respectively.)

Background. The development of an effective human immunodeficiency virus (HIV) vaccine is a high global priority. Here, we report the safety, tolerability, and immunogenicity of a replication-defective recombinant adenovirus serotype 5 (rAd5) vector HIV-1 candidate vaccine.

Methods. The vaccine is a mixture of 4 rAd5 vectors that express HIV-1 subtype B Gag-Pol fusion protein and envelope (Env) from subtypes A, B, and C. Healthy, uninfected adults were randomized to receive 1 intramuscular injection of placebo ($n = 6$) or vaccine at dose levels of 10^9 ($n = 10$), 10^{10} ($n = 10$), or 10^{11} ($n = 10$) particle units and were followed for 24 weeks to assess immunogenicity and safety.

Results. The vaccine was well tolerated but was associated with more reactogenicity at the highest dose. At week 4, vaccine antigen–specific T cell responses were detected in 28 (93.3%) and 18 (60%) of 30 vaccine recipients for $CD4^+$ and $CD8^+$ T cells, respectively, by intracellular cytokine staining assay and in 22 (73%) of 30 vaccine recipients by enzyme-linked immunospot assay. Env-specific antibody responses were detected in 15 (50%) of 30 vaccine recipients by enzyme-linked immunosorbant assay and in 28 (93.3%) of 30 vaccine recipients by immunoprecipitation followed by Western blotting. No neutralizing antibody was detected.

Conclusions. A single injection induced HIV-1 antigen–specific $CD4^+$ T cell, $CD8^+$ T cell, and antibody responses in the majority of vaccine recipients. This multiclade rAd5 HIV-1 vaccine is now being evaluated in combination with a multiclade HIV-1 DNA plasmid vaccine.

More than 40 million people are living with HIV/AIDS. Three million deaths occur annually because of HIV/AIDS, with ~5 million new infections occurring in 2005 [1]. Development of an effective vaccine would be an important intervention to help control the expanding global pandemic.

Adenovirus serotype 5 (Ad5) has been developed as a replication-defective recombinant vector (rAd5) to deliver intracellular genes via a number of routes [2]. Vaccination with rAd5 results in transient intracellular gene expression followed by rapid clearance [3]. Cellular and humoral immune responses have been induced in preclinical studies of rAd5 vaccines for HIV-1, simian immunodeficiency virus (SIV), and simian-human immunodeficiency virus (SHIV) [4–7]. This approach builds on previous successes with other infectious disease models, particularly for Ebola virus, against which nonhuman primates have been protected

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Potential conflicts of interest: G.J.N. and B.K.C. are named on patent applications for this vaccine concept. B.T.B., J.G.D.G., and C.R.K. are employees of GenVec, Inc. All other authors report no conflicts of interest.

Financial support: National Institute of Allergy and Infectious Diseases intramural research program.

Clinicaltrials.gov registry number: NCT00083330.

* Study group members are listed after the text.

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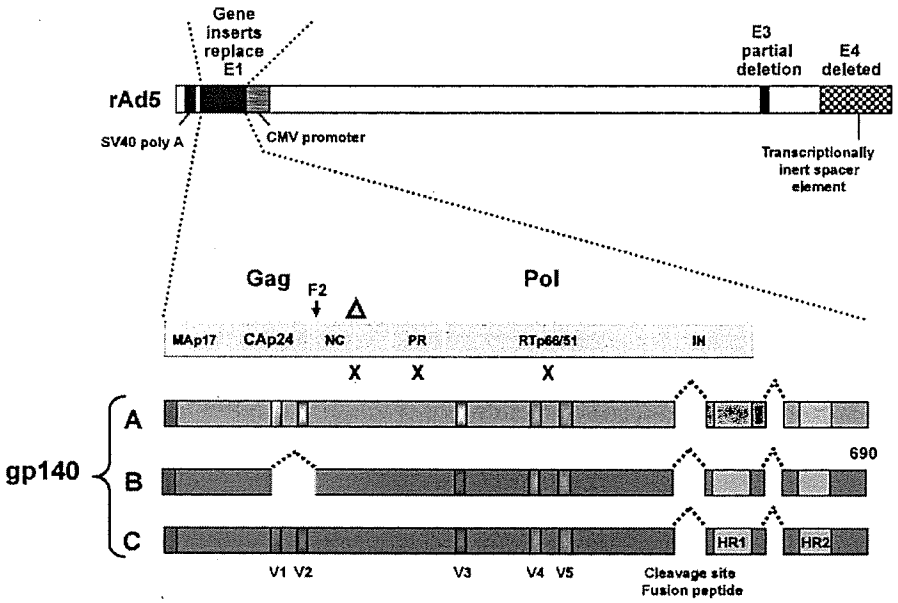

Figure 1. Schematic of the design of the replication-defective recombinant adenovirus serotype 5 (rAd5) vector vaccine. Four separate rAd5 vectors were produced using the same genetic backbone and manufacturing approaches. The HIV-1 vaccine antigen–expression cassettes in the E1 region contained the immediate-early cytomegalovirus (CMV) enhancer/promoter (GenBank accession no. X17403; nucleotide positions 174314–173566), positioned right to left with respect to the viral E1 region. This was followed by an artificial untranslated region of 144 bp and 3′ splice-site sequences, the open reading frame (ORF) of the gene to be expressed, and the simian virus 40 polyadenylation signal (SV40 poly A). The E4 region and a portion of the E3 region were also deleted as shown. A fusion Gag/Pol polyprotein was encoded by a synthetic ORF with nucleotide sequences based on the *gag* gene from clade B strain HXB2 (GenBank accession no. K03455) and the *pol* gene *(pol/h)* from clade B strain NL4-3 (GenBank accession no. M19921). Mutations (indicated by Xs), including the deletion of the carboxy-terminus of Gag (indicated by the triangle), were introduced in the protease and reverse-transcriptase genes to prevent processing of the *pol* gene products, reducing the potential for functional enzymatic activity [12]. This resulted in a fusion protein that directly reads through the frame shift in Gag (F2). To create synthetic gp140 versions of the Env genes truncated at the transmembrane domain of gp41, sequences from clade A strain 92rw020 (CCR5 tropic; GenBank accession no. U08794), clade B strain HXB2 (X4 tropic; GenBank accession no. K03455) with V1 and V2 deleted and V3 replaced with BaL sequence (GenBank accession no. M68893), and clade C strain 97ZA012 (CCR5 tropic; GenBank accession no. AF286227) were used [13]. In each construct, the cleavage site and fusion peptide at the junction of gp120 and gp41 was deleted, and a portion of the interspace between the 2 heptad-repeat regions in gp41 was deleted. In addition, deletion of the V1/V2 loops from the EnvB construct was required to improve the stability of the vector during manufacturing. HR1–HR2, heptad-repeat regions in gp41; IN, integrase; NC, nucleocapsid; PR, protease; V1–V5, variable regions in envelope.

from lethal challenge [8, 9]. Recently, immunization of chimpanzees with rAd expressing hepatitis C virus (HCV) nonstructural genes resulted in T cell–mediated protection against heterologous HCV challenge [10]. These preclinical studies support the concept that gene-based vaccination can induce effective immunity against viral infections in primates.

The development of an HIV vaccine that is effective against multiple circulating viral clades remains a scientific priority and urgent public health need [11]. The rAd5 vaccine evaluated in the present clinical trial was designed to express an HIV-1 clade B Gag-Pol fusion protein and Env glycoproteins from HIV-1 clades A, B, and C. Here, we report the findings from the first phase 1 clinical trial of this multigene, multiclade rAd5 HIV-1 candidate vaccine.

SUBJECTS, MATERIALS, AND METHODS

Study design. Vaccine Research Center (VRC) 006 (National Institutes of Health [NIH] 04-I-0172) was a randomized, double-blinded, placebo-controlled phase 1 trial conducted at the NIH Clinical Center (Bethesda, MD) by the VRC (National Institute of Allergy and Infectious Diseases [NIAID], NIH, Department of Health and Human Services). Enrollment began 19 July 2004, and unblinding occurred on 27 May 2005. Eligibility criteria required that volunteers be HIV uninfected, 18–44 years old, amenable to risk-reduction counseling, and in good general health as determined by medical history, physical examination, and laboratory tests. Thirty-six volunteers were enrolled into 3 dose groups of 12 study subjects and were randomized to receive vaccine or placebo at a 5:1 ratio. Vaccine doses of 10^9, 10^{10}, and 10^{11} particle units (PUs; $n = 10$ subjects/group) and injection of the final formulation buffer as placebo

Table 1. Subject demographics.

Category, parameter	Vaccine recipients (n = 30)	Placebo recipients (n = 6)	All subjects (n = 36)
Sex			
Male	15 (50)	5 (83.3)	20 (55.6)
Female	15 (50)	1 (16.7)	16 (44.4)
Age			
18–20 years	0 (0)	1 (16.7)	1 (2.8)
21–30 years	19 (63.3)	5 (83.3)	24 (66.7)
31–44 years	11 (36.7)	0 (0)	11 (30.6)
Mean ± SD, years	28.2 ± 6.4	23.5 ± 2.4	27.4 ± 6.2
Race			
White	23 (76.7)	4 (66.7)	27 (75)
Black or African American	3 (10)	2 (33.3)	5 (13.9)
Asian	2 (6.7)	0 (0)	2 (5.6)
American Indian/Alaskan Native	0 (0)	0 (0)	0 (0)
Multiracial	2 (6.7)	0 (0)	2 (5.6)
Ethnicity			
Non-Hispanic/Latino	28 (93.3)	5 (83.3)	33 (91.7)
Hispanic/Latino	2 (6.7)	1 (16.7)	3 (8.3)

NOTE. Data are no. (%) of subjects, unless otherwise indicated.

(n = 6 subjects) were evaluated. A 1-mL intramuscular (deltoid) injection was administered on the day of enrollment. The NIAID Data Safety and Monitoring Board completed a safety review before each dose escalation. Safety evaluations included physical examination and monitoring of laboratory parameters. Local (pain, swelling, or redness) and systemic (fever, malaise, myalgia, headache, chills, or nausea) reactogenicity symptoms after vaccination were recorded on a 5-day diary card. Adverse events were graded for severity by use of a preapproved table that incorporated a 5-point scale and were coded by use of Medical Dictionary for Regulatory Activities terminology.

To address a theoretical concern about the interaction of rAd5 with a naturally acquired adenovirus, subjects experiencing upper respiratory tract infection, urinary tract infection, gastroenteritis, or conjunctivitis within 4 weeks of the study injection had a specimen collected for adenoviral cultures. Specimens were cultured for 5 days via shell vial on a human lung epithelial cell line monolayer (Diagnostic Hybrid) and were screened for known adenovirus serotypes by an indirect fluorescent antibody staining method (VRK Bartels Viral Respiratory Screening and Identification Kit, Bartels).

Vaccine. The VRC-HIVADV014-00-VP vaccine is a 3:1:1:1:1 ratio of recombinant adenovirus vectors that encode for HIV-1 subtype B Gag-Pol fusion protein and Env glycoproteins from clades A, B, and C, respectively. The transgenes were developed at the VRC, and the design is shown in detail in figure 1. Protein expression was optimized by using preferential amino acid sequences found in human cells.

The transgenes were inserted into the GV11 (GenVec) adenoviral vector system, which is based on human serotype 5 and contains deletions of the E1 and E4 regions and part of the E3 region, rendering it replication defective. The vectors were as described elsewhere [14], except in the expression cassette contained in the E1 region (figure 1). The vector stocks were serially passaged on complementing mammalian cells (293-ORF6), to generate high-titer stocks of replication-defective adenoviruses [15, 16]. The absence of replication-competent adenovirus was verified by the product-release assays. The adenovirus vectors were purified from the cell substrate by a cesium chloride gradient centrifugation process, dialyzed into final formulation buffer, diluted to the desired concentration, and pooled to form the final vaccine product.

Clinical trial material was manufactured under contract at Molecular Medicine BioServices, under current good manufacturing practice conditions. The Vaccine Clinical Materials Program (operated by Science Applications International Corporation) provided quality-assurance oversight of clinical production and release. In compliance with current US Food and Drug Administration guidance, the vaccine was tested for safety, purity, potency, identity, and quality before release. Placebo consisted of the final formulation buffer, which was custom manufactured by Cambrex.

Flow-cytometric analysis and enzyme-linked immunospot (ELISpot) assays. The frequency of vaccine antigen–specific cells was determined as described elsewhere [17]. Cryopre-

Table 2. Local reactogenicity.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

Table 3. Self-assessed maximum systemic reactogenicity summary.

Systemic symptoms, intensity	Vaccine recipients				Placebo recipients (n = 6)
	10^9 PUs (n = 10)	10^{10} PUs (n = 10)	10^{11} PUs (n = 10)	All (n = 30)	
Malaise					
None	10 (100)	6 (60)	2 (20)	16 (53.3)	6 (100)
Mild	0 (0)	4 (40)	4 (40)	8 (26.7)	0 (0)
Moderate	0 (0)	0 (0)	4 (40)	4 (13.3)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Myalgia					
None	10 (100)	7 (70)	4 (40)	21 (70)	5 (83.3)
Mild	0 (0)	3 (30)	3 (30)	6 (20)	1 (16.7)
Moderate	0 (0)	0 (0)	3 (30)	3 (10)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Headache					
None	8 (80)	7 (70)	4 (40)	19 (63.3)	4 (66.7)
Moderate	0 (0)	0 (0)	4 (40)	4 (13.3)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Chills					
None	10 (100)	10 (100)	6 (60)	26 (86.7)	6 (100)
Mild	0 (0)	0 (0)	3 (30)	3 (10)	0 (0)
Moderate	0 (0)	0 (0)	1 (10)	1 (3.3)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nausea					
None	9 (90)	8 (80)	6 (60)	23 (76.7)	5 (83.3)
Mild	1 (10)	2 (20)	3 (30)	6 (20)	1 (16.7)
Moderate	0 (0)	0 (0)	1 (10)	1 (3.3)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fever					
None	10 (100)	10 (100)	6 (60)	26 (86.7)	6 (100)
Mild	0 (0)	0 (0)	3 (30)	3 (10)	0 (0)
Moderate	0 (0)	0 (0)	1 (10)	1 (3.30)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Any systemic symptom					
None	8 (80)	4 (40)	1 (10)	13 (43.3)	4 (66.7)
Mild	2 (20)	6 (60)	3 (30)	11 (36.7)	2 (33.3)
Moderate	0 (0)	0 (0)	6 (60)	6 (20)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

NOTE. Data are no. (%) of subjects. Systemic reactogenicity, reported by subjects on diary cards, is listed by dose group. PUs, particle units.

served peripheral-blood mononuclear cells (PBMCs) were stimulated by use of peptide pools (15mer overlapping by 11 aa) representing the individual vaccine antigens (6 h with brefeldin A for the intracellular cytokine staining [ICS] assay and overnight for the ELISpot assay). Fixed, permeabilized cells were evaluated by flow cytometry for expression of CD3, CD4, CD8, and interferon (IFN)–γ and/or interleukin (IL)–2 and then analyzed using FlowJo software (Tree Star Software). IFN-γ ELISpot assays were performed using a commercial kit (BD Biosciences); results were read using a CTL ELISpot image analyzer (Cellular Technology) and are expressed as the mean number of spot-forming cells per 10^6 PBMCs.

Measurement of antibody responses. Standardized research ELISAs were performed to delineate the antibody response to viral antigens encoded in the vaccine. End-point titers of antibodies were determined using 96-well Immulon 2 plates (Dynex Technologies) coated with a preparation of purified recombinant HIV proteins [17]. The end-point titer was calculated as the most dilute serum concentration that gave an optical density reading >0.2 above background. Analysis by immunoprecipitation followed by Western blotting (IP–Western blotting) [13], HIV-1 neutralization [18], and Ad5 neutralization [19] was done. Subjects were screened using a commercial ELISA (Abbott Laboratories HIV-1/HIV-2 rDNA) and

Table 4. Commercial assay results for HIV-1 antibody at study week 24.

	ELISA		Western blot			
Group	Negative	Positive	Negative	Indeterminate	Positive	Uninterpretable
Placebo (n = 6)	6	0	...	...	...	...
10^9 PUs (n = 10)	7	3	2	0	0	1
10^{10} PUs (n = 10)	4	6	2	4	0	0
10^{11} PUs (n = 10)	1	9	1	4	4	0

NOTE. The Abbott HIVAB HIV-1/HIV-2 rDNA kit was used for routine commercial HIV ELISA testing. Western blot analysis was performed only if the ELISA result was positive. The Western blot analyses were done at the Mayo Laboratory by use of the Genetic Systems HIV Western Blot kit (BioRad Laboratories). A positive Western blot required a band at p24 in addition to a band for at least 1 of the Env glycoproteins (gp41, gp120, or gp160). If there were bands present that did not meet the positivity criteria, the result was reported as indeterminate. If a blot had a high level of nonspecific background staining, it was reported as uninterpretable. HIV-1 infection did not occur in any study subjects, as confirmed by polymerase chain reaction. PUs, particle units.

Western blotting (Genetic Systems HIV Western blot kit; Bio-Rad Laboratories; performed at the Mayo Laboratory, Rochester, MN).

Statistical methods. T cell data are summarized by positive response rates to individual peptide pools and exact 2-sided 95% confidence intervals (CIs). Positivity criteria for ICS and ELISpot data consisted of a statistical hypothesis test for a difference between stimulated and unstimulated wells and a minimal level of response requirement (i.e., the difference had to be statistically significant and exceed a threshold). For ICS responses, Fisher's exact test (α = .01) was applied to each antigen-specific response versus the negative control response, with a Holm adjustment for multiplicity. The minimum threshold for background-corrected positive-response percentage was 0.0241% for $CD4^+$ T cells and 0.0445% for $CD8^+$ T cells. These thresholds were selected to give a 1% false-positive rate in a VRC ICS validation study that included 34 HIV-1–seronegative individuals and that used 8 HIV peptide pools; only 2 (0.007%) of 272 individuals had responses exceeding the thresholds. For ELISpot responses, a permutation test (α = .05) was applied using the Westfall-Young approach to adjust for multiplicity [20]. The threshold was 50 sfc/10^6 PBMCs. A variance filter for the antigen-specific responses was also used: samples with a ratio of antigen-well variance to (median + 1) of $\geq$100 were excluded; no such samples were found. SAS (version 9.1; SAS Institute) and Splus (version 6.0; Insightful) were used for all analyses.

RESULTS

Study population. The mean age of the study participants was 27.4 years (SD, 6.2 years); 56% were men, and 44% were women (table 1). All subjects received the study injection, completed the diary card, and completed 24 weeks of clinical observation. Before vaccination, none of the placebo recipients had a preexisting Ad5 antibody titer $\geq$1:12, whereas 16 of the 30 vaccine recipients were seropositive for Ad5 antibody: 7 in the 10^9-PU group, 6 in the 10^{10}-PU group, and 3 in the 10^{11}-PU group.

Vaccine safety. Local and systemic signs and symptoms (i.e., reactogenicity) increased in frequency and severity with vaccine dose but were never of more than moderate (grade 2) severity. Local reactogenicity was reported by 2 (20%) of the 10 vaccine recipients in the 10^9-PU group, 8 (80%) of the 10 vaccine recipients in the 10^{10}-PU group, and 10 (100%) of the 10 vaccine recipients in the 10^{11}-PU group; by comparison, 2 (33%) of the 6 placebo recipients reported having at least 1 local symptom. Pain was the most frequently reported symptom; all local reactogenicity was recorded as being of mild (grade 1) severity, except for 1 report of moderate local pain by a vaccine recipient who received the 10^{11}-PU dose (table 2). Systemic reactogenicity was reported by 2 (20%) of the 10 vaccine recipients in the 10^9-PU group, 6 (60%) of the 10 vaccine recipients in the 10^{10}-PU group, and 9 (90%) of the 10 vaccine recipients in the 10^{11}-PU group; by comparison, 2 (33%) of the 6 placebo recipients reported having at least 1 systemic symptom. Systemic symptoms did not exceed mild severity at the 10^9- and 10^{10}-PU doses, and all vaccine recipients remained afebrile. At the 10^{11}-PU dose, 6 (60%) of the 10 vaccine recipients reported having at least 1 systemic symptom of moderate severity (table 3). Of these 6, 4 reported having fever (3 mild and 1 moderate in severity) beginning 18–24 h after injection. These 4 vaccine recipients reported having moderate headache 1 day after vaccination, and 3 of these vaccine recipients reported having at least 1 other moderate systemic symptom (malaise, myalgia, or chills). The fevers resolved within 24 h, and other symptoms decreased in severity within a day, although mild symptoms persisted for up to 4 days in some subjects.

Other adverse events that occurred after vaccination were either mild or moderate in severity except for a seizure that occurred 64 days after vaccination. This event was assessed as being unrelated to vaccination, on the basis of the timing and of a

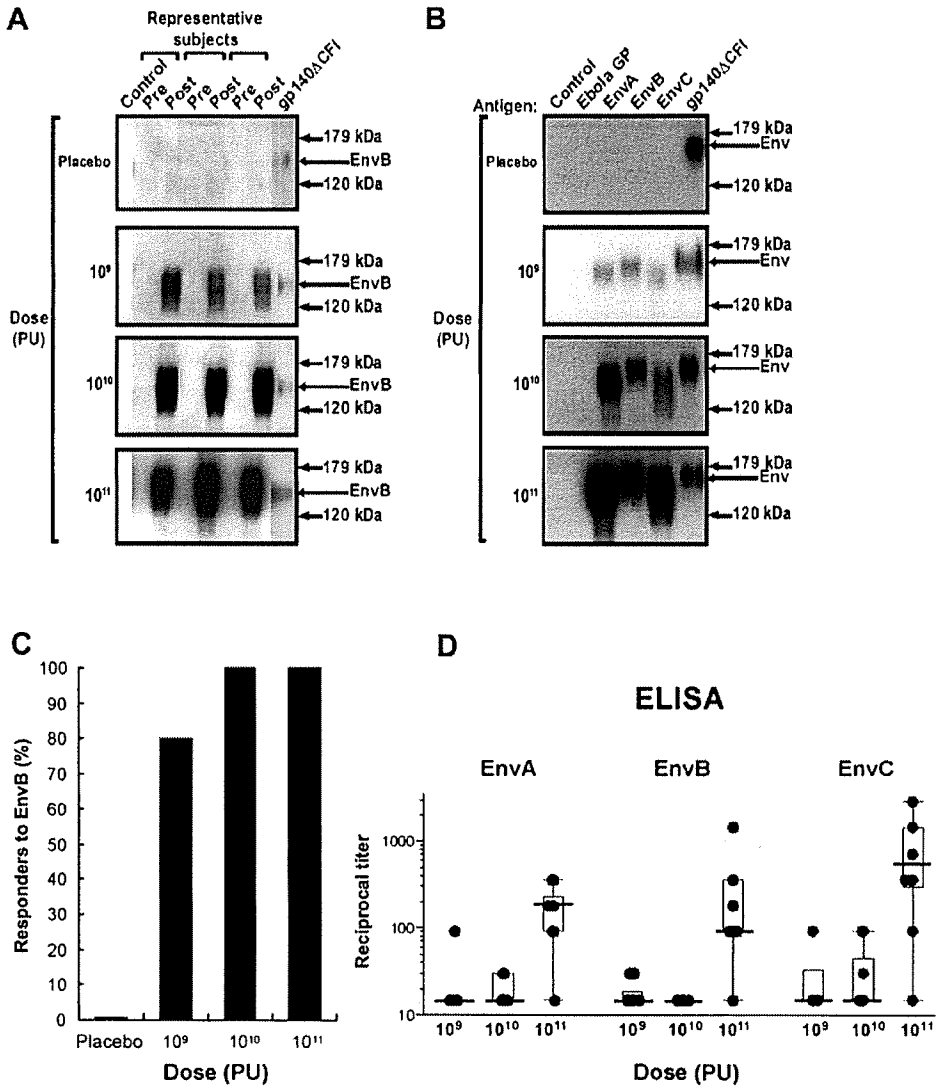

Figure 2. Induction, specificity, and dose response of vaccine-induced antibody to clades A, B, and C Env proteins. *A,* Western blot analysis of the antigen captured by immunoprecipitation using prevaccination (pre) and week 4 (post) serum samples from 3 representative subjects in the placebo group and in the 3 dose groups receiving 10^9, 10^{10}, or 10^{11} particle units (PUs). The analysis shows that Env-specific antibody is induced by the vaccine; arrows indicate the EnvB-specific band. *B,* Recognition of vaccine-induced antibody. Serum from 1 subject representing each of the dose groups shows that vaccine-induced antibody recognized all 3 Env subtypes but not the Ebola virus glycoprotein–negative control (Ebola GP). Arrows indicate the Env-specific band. No positive bands were detected in any of the placebo recipients at any time point in the study. *C,* Frequency of positive antibody responders to EnvB at week 4 as measured by immunoprecipitation followed by Western blotting, for each dose group. *D,* Geometric means of the reciprocal dilution of antibody to purified gp140 for EnvA, EnvB, and EnvC for each subject at week 4, by dose group. Titers were determined by end-point titration ELISA. Error bars represent SDs. The dilution series began at 1:30, and negative samples were assigned a value of 1:15. The proteins used for ELISA were between 85% and 90% pure as determined by Western blotting and polyacrylamide gel electrophoresis.

medical history of a seizure (3 years prior). There were 3 grade 2 adverse events assessed as being possibly related to vaccination, as follows: (1) asymptomatic neutropenia occurring 21 days after vaccination in a subject in the 10^9-PU group who had had documented transient mild neutropenia before vaccination; (2) an episode of diarrhea (1-day duration) occurring 3 days after vaccination in a subject in the 10^{11}-PU group; and (3) steatohepatitis (fatty liver) in a subject in the 10^{11}-PU group that was diagnosed by ultrasound after observation of a persistent grade 1 elevated alanine aminotransferase level noted 25 days after vaccination and lasting for ~5 months. This last event was assessed by a hepatologist as most likely being related to recent rapid weight gain and alcohol consumption.

Eight specimens were obtained from 6 vaccine recipients after

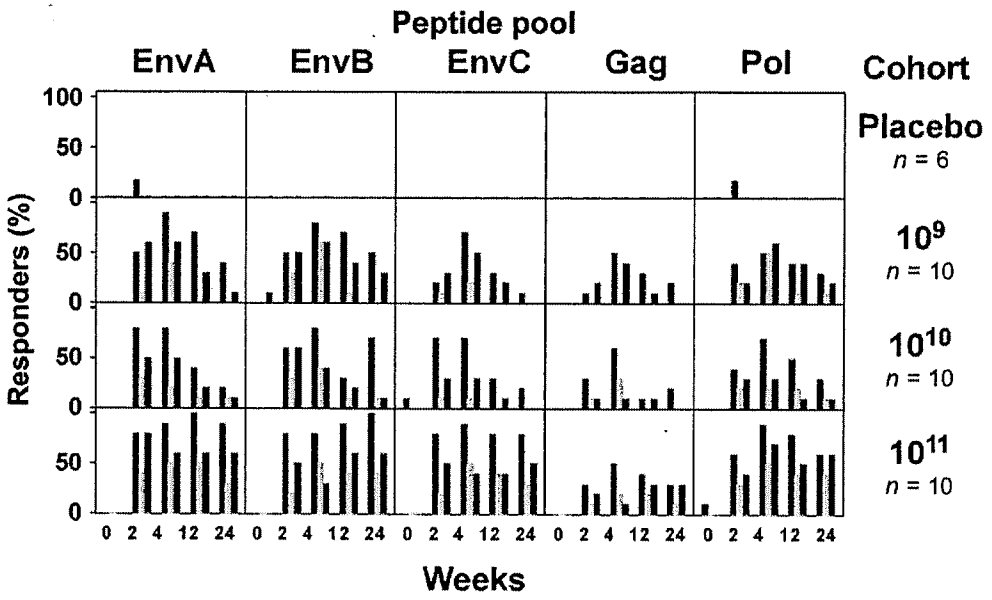

Figure 3. Frequency of subjects with detectable T cell responses. T cell responses to each peptide pool in all dose groups are shown. Each box shows the entire time course for each T cell assay. The *Y*-axis of each box shows the frequency of positive responders to the respective peptide pool for each assay as percentage of subjects in a dose group (0%–100%). Red bars indicate $CD4^+$ T cell responses as measured by intracellular cytokine staining (ICS) assay, green bars indicate $CD8^+$ T cell responses as measured by ICS assay, and blue bars indicate $CD4^+$ or $CD8^+$ T cell responses as measured by enzyme-linked immunospot assay (ELISpot).

the onset of upper respiratory tract infections (pharynx; n = 7) or a urinary tract infection (urine; n = 1) for adenovirus culture. All cultures were negative for adenovirus. A stool sample could not be obtained from 1 subject with transient diarrhea.

Overall, 18 (60%) of the 30 vaccine recipients had vaccine-induced HIV-1 antibody detected by commercial ELISA at the first postvaccination testing time point (week 12), and all antibody responses persisted through week 24. The frequency of diagnostic ELISA positivity increased with vaccine dose: 3 (30%) of 10 in the 10^9-PU group, 6 (60%) of 10 in the 10^{10}-PU group, and 9 (90%) of 10 in the 10^{11}-PU group (table 4). Western blots were indeterminate or positive at week 24 in 0 (0%) of the 10 vaccine recipients in the 10^9-PU group, 4 (40%) of the 10 vaccine recipients in the 10^{10}-PU group, and 8 (80%) of the 10 vaccine recipients in the 10^{11}-PU group.

Vaccine-specific antibody responses. At week 4, IP-Western blotting (figure 2*A* and 2*B*) indicated that 28 (93.3%) of the 30 vaccine recipients had developed antibody to EnvB (figure 2*C*). By research ELISAs, 15 (50%) of the 30 vaccine recipients had developed an antibody response to 1 or more vaccine antigen by week 4, with the greatest frequency of response being to EnvC. The geometric mean reciprocal titer of antibody to Env in the 10^{11}-PU group at week 4 among responders was 200, 310, and 1010, for EnvA, EnvB, and EnvC, respectively (figure 2*D*). The ELISA titer for each Env antigen was significantly higher at the 10^{11}-PU dose than at either the 10^9- or 10^{10}-PU dose ($P < .001$, Wilcoxon rank sum test). Weak antibody responses to Gag were noted in 1 (10%) of the 10 vaccine recipients in the 10^{10}-PU group and 5 (50%) of the 10 vaccine recipients in the 10^{11}-PU group. There was a greater magnitude of antibody response to EnvC than to EnvA (P = .022, Wilcoxon rank sum test) and a trend toward greater responses to both of these antigens compared with responses to EnvB (P = .051, Wilcoxon rank sum test).

No HIV-1–specific neutralizing antibody was detected. Serum samples obtained from vaccine recipients at week 4 and week 24 after vaccination and diluted 1:10 did not neutralize viruses HXB2 or SF162.

Vaccine-induced T cell responses. Antigen-specific $CD8^+$ T cell responses were detected 2 weeks after vaccination in most vaccine recipients and peaked at week 4 (figure 3). T cell responses were observed by both ICS assay (IFN-γ and/or IL-2) and ELISpot assay (IFN-γ). The Env antigens elicited the most frequent response (figure 4*A* and 4*B*).

Twenty-eight (93.3% [95% CI, 77.9%–99.2%]) of the 30 vaccine recipients had a positive $CD4^+$ T cell response to at least 1 Env peptide pool by week 4. Twenty-seven (90% [95% CI, 73.5%–97.9%]) of the 30 had a $CD4^+$ T cell response to at least 1 Env peptide pool, 15 (50% [95% CI, 31.3%–68.7%]) of the 30 had responses to Gag, and 16 (53.3% [95% CI, 34.3%–71.7%]) of the 30 had responses to the Pol peptide pools by week 4 (figure 3). The magnitude of $CD4^+$ T cell responses ranged from 0.0013% to 0.15%, and the median response peaked at week 4 (figure 4*A*). The magnitude of the peak re-

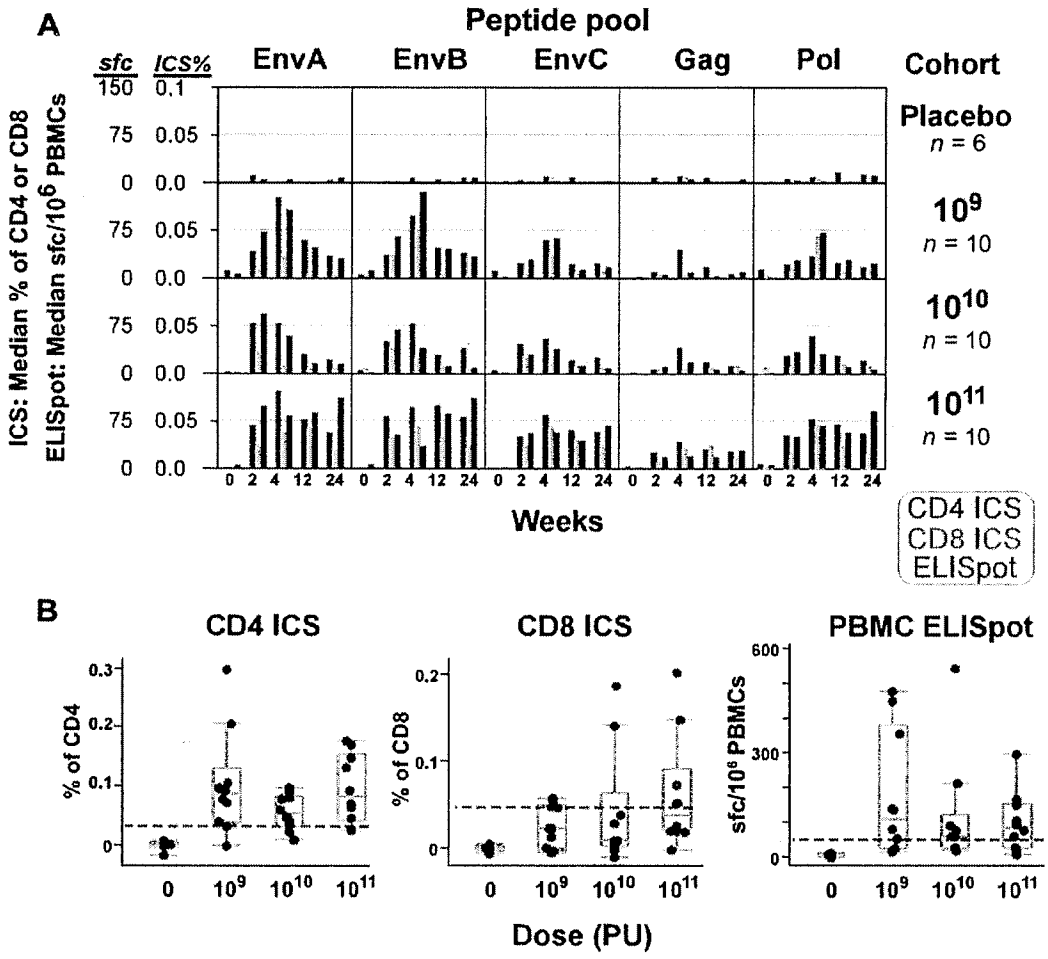

Figure 4. Magnitude of T cell responses to specific vaccine components. T cell responses to each peptide pool for each dose group were measured by intracellular cytokine staining (ICS) assay to detect interferon (IFN)–γ and/or interleukin-2 and by IFN-γ enzyme-linked immunospot (ELISpot) assay for all placebo and vaccine recipients. *A,* Median magnitudes of peptide pool–specific responses, shown as a percentage of total $CD4^+$ or $CD8^+$ T cells for the ICS assay (scale 0–0.1) and as the no. of spot-forming cells per 10^6 peripheral-blood mononuclear cells (PBMCs) for the ELISpot assay (scale 0–150), by dose group. Both values are plotted on a linear scale. Each box shows the 24-week time course of the study for each T cell assay. Red bars indicate $CD4^+$ T cell responses as measured by ICS assay, green bars indicate $CD8^+$ T cell responses as measured by ICS assay, and blue bars indicate $CD4^+$ or $CD8^+$ T cell responses as measured by ELISpot assay. *B,* Magnitudes of EnvA-specific T cell responses at study week 4 for each subject as measured by 3 assays. Shown are the percentages of $CD4^+$ or $CD8^+$ T cells producing cytokine as measured by ICS assay and the no. of spot-forming cells per 10^6 PBMCs as measured by ELISpot assay, by dose group. The box plots indicate the median, 25th, and 75th percentiles for each dose level, and the error bars show the 5th and 95th percentile. The horizontal dashed line on each plot indicates the threshold of positivity.

sponse did not appear to be affected by vaccine dose (figure 4*B*). However, at week 24, there was a trend toward a dose effect with respect to the persistence of $CD4^+$ T cells responses (table 5).

At week 4, 20 (66.7% [95% CI, 47.2%–82.7%]) of the 30 vaccine recipients had a $CD8^+$ T cell response stimulated by at least 1 Env peptide pool, 5 (16.7% [95% CI, 5.6%–34.7%]) of the 30 had responses to Gag, and 12 (40% [95% CI, 22.7%–59.4%]) of the 30 had responses to the Pol peptide pools (figure 3). The magnitude of the $CD8^+$ T cell responses ranged from 0.0019% to 0.69%, and the median response peaked at week 4 (figure 4*A*). By week 24, there was a trend toward better persistence of $CD8^+$ T cell responses in the highest dose group (table 5).

ELISpot responses (the IFN-γ end point in unfractionated PBMCs) peaked at week 4, with 22 (73.3%) of the 30 vaccine recipients showing a response to 1 or more of the vaccine-specific peptide pools. The frequency of ELISpot responses to specific peptide pools at week 4 were 20 (66.7%) of 30 for EnvA, 14 (46.7%) of 30 for EnvB, 5 (50%) of 30 for EnvC, 10 (33%) of 30 for Pol-1, 9 (30%) of 30 for Pol-2, and 7 (23.3%) of 30 for Gag (figure 3). The geometric (background-corrected)

Table 5. Frequency of subjects with positive T cell responses to the dose levels of 10^9 particle units (PUs), 10^{10} PUs, and 10^{11} PUs at 4 and 24 weeks after vaccination, as measured by intracellular cytokine staining (ICS) assay in $CD4^+$ and $CD8^+$ T cells and by enzyme-linked immunospot (ELISpot) assay in $CD4^+$ or $CD8^+$ T cells.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

means at week 4 for all dose groups combined, in order of magnitude, were 68 sfc/10^6 PBMCs for EnvA, 60 sfc/10^6 PBMCs for EnvB, 49 sfc/10^6 PBMCs for EnvC, 23 sfc/10^6 PBMCs for Pol-1, 23 sfc/10^6 PBMCs for Pol-2, and 13 sfc/10^6 PBMCs for Gag. The magnitude (figure 4*A*) and frequency (table 5) of antigen-specific ELISpot responses were consistent with the pattern observed by ICS assay.

The overall magnitude of the T cell responses and the kinetics of the responses for $CD4^+$ and $CD8^+$ T cells show that the peak responses occurred during week 4 after vaccination (figure 5*A*). The magnitude of the ELISpot response in the 10^{11}-PU group was maintained through the 24 weeks of the study.

Vaccine recipients who were seropositive for Ad5 antibody before vaccination frequently mounted cellular responses to the vaccine antigens (figure 5). However, evaluation of the number of total spot-forming cells (sum of highest Env response plus the Gag and the Pol responses) revealed a significant effect of preexisting Ad5 neutralizing antibody for all rAd5 doses at weeks 4, 12, and 24 ($P < .002$ for all, linear mixed effects model). Overall, for subjects with a preexisting reciprocal 90% Ad5 neutralization titer <1:12, the total ELISpot response was 3.29 times higher than that in subjects who were seropositive for Ad5 antibody (figure 5*B*).

DISCUSSION

The induction of significant T cell and antibody responses in nonhuman primates by use of a multiclade rAd5 prototype and the conferment of protection from SHIV and SIV challenge provided the rationale for the present clinical trial. rAd5 vectors have several attractive features, including (1) scalable manufacturing in stable cell lines approved for other human biologics; (2) efficient gene delivery to antigen-presenting cells [21]; (3) potent transient protein expression; (4) induction of innate immune responses; (5) induction of antibody responses; (6) intracellular production of antigen, allowing induction of both $CD8^+$ and $CD4^+$ T cell responses [22]; and (7) induction of immunity after a single injection.

The present study identified a delivery approach and dose of a candidate multiclade HIV-1 rAd5 vaccine that was assessed as safe and immunogenic. Vaccination did not cause severe adverse reactions. The highest dose of rAd5 caused a moderate, short-lived syndrome of headache, myalgia, malaise, and fever that began within 24 h after vaccination. Therefore, it is expected that dose-dependent systemic reactogenicity may follow rAd5 vaccination.

The induction of HIV-specific cellular immunity is a major goal for HIV vaccine development. HIV-specific $CD8^+$ T cell responses clear virus-infected cells and appear during the declining viremia that follows acute infection [23, 24]. High levels of $CD8^+$ T cells present during chronic HIV-1 infection suggest their importance in the control of viremia [25–34]. HIV-specific $CD4^+$ T cell responses also peak early during infection; however, this response diminishes soon after seroconversion [35, 36]. Maintenance of a functional HIV-specific $CD4^+$ T cell response correlates with long-term nonprogression of HIV disease [37]. In addition, nonhuman primate models of lentivirus infection have shown that either $CD8^+$ T cell depletion or mutation of key $CD8^+$ T cell epitopes within the circulating virus results in increased levels of viremia and disease progression [32, 38]. These observations suggest that HIV-1–specific $CD4^+$ and $CD8^+$ T cells are important for controlling HIV replication and preventing disease progression.

The present study demonstrates that a single rAd5 immunization can induce HIV-specific $CD4^+$ and $CD8^+$ T cell responses in the majority of vaccinated subjects (response rates by week 24 were 28/30 [93.3%] and 20/30 [60%], respectively). The peak cellular response occurred 4 weeks after vaccination, and the frequency of detectable responses diminished during the 24-week follow-up period. The peak cellular immune response was not dose related, although there was a greater frequency of responses in the higher dose groups and greater frequency of detectable cellular responses for 24 weeks in the 10^{11}-PU group. The frequency and magnitude of both $CD4^+$ and $CD8^+$ T cell responses to rAd5 immunization as measured by the ICS and ELISpot assays were similar to those induced by 3 doses of plasmid DNA vaccine expressing matching vaccine antigens [17]. Defining the duration of response more precisely will require larger studies, which are currently under way.

Generating a neutralizing antibody response to HIV-1 may be the greatest challenge to the development of a protective HIV vaccine [39]. Although rAd5 vaccination induced modest levels of HIV-specific antibodies as measured by ELISA (~10-fold higher magnitude than in DNA-primed subjects [17]), it did not induce significant HIV-1 neutralizing antibodies to HXB2 or SF162. Typically, neutralizing activity is not detected in the serum of HIV-1–infected humans or SIV-infected macaques until the ELISA titer is >1:100,000. By ELISA, there was a greater frequency and titer of HIV-specific antibodies in the 10^{11}-PU group (figure 2*D*) than in the 10^9- or 10^{10}-PU group, suggesting a threshold effect for antibody induction. The magnitude of antibody responses to EnvC was greater than that to EnvA ($P = .022$). However, it is not known whether this was

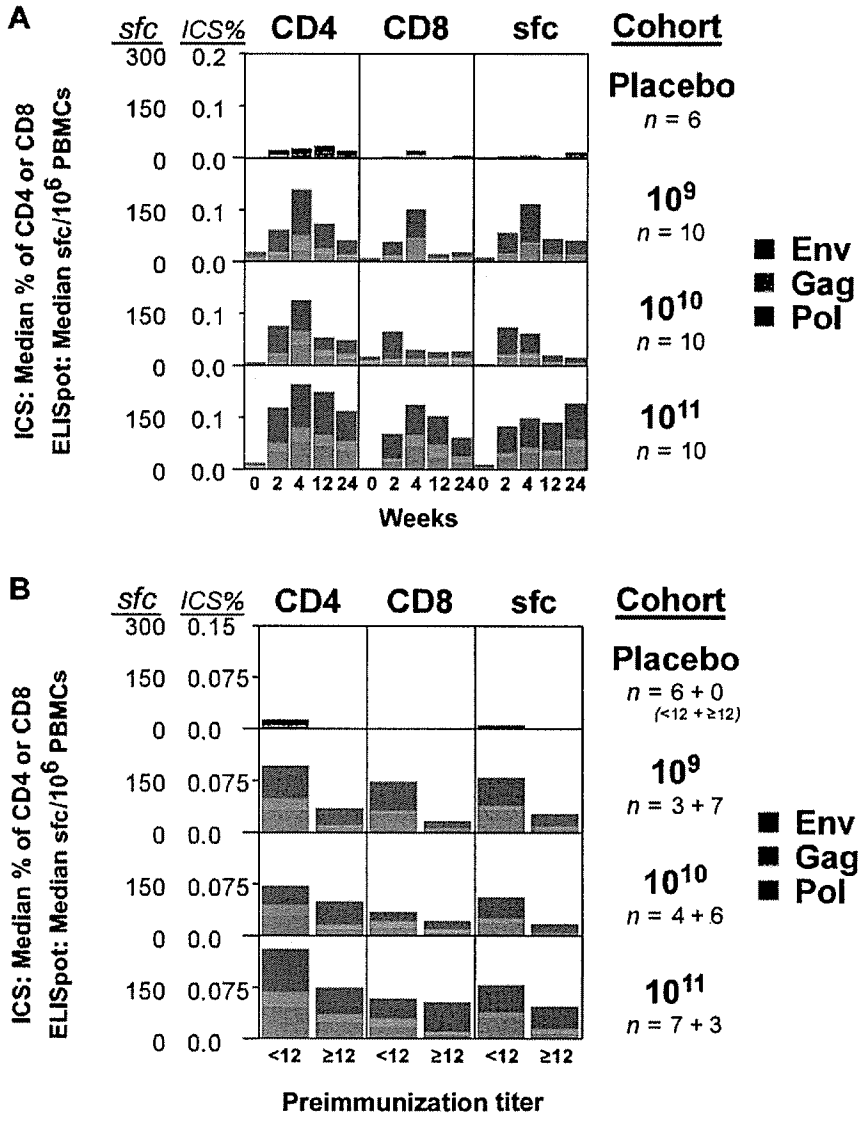

Figure 5. Combined cellular responses to vaccine antigens. The highest Env response to a single subtype was added to the Gag and Pol responses for each subject as a measure of the total vaccine-induced T cell response, and the median for each dose group was plotted on a linear scale for the intracellular cytokine staining (ICS) and the enzyme-linked immunospot (ELISpot) assays. *A*, Median magnitudes for maximum Env *(blue)*, Gag *(grey)*, and Pol *(brown)* are shown as a percentage of total $CD4^+$ or $CD8^+$ T cells producing cytokine for the ICS assay and as the no. of spot-forming cells per 10^6 peripheral-blood mononuclear cells (PBMCs) for the ELISpot assay. Each box represents the 24-week time course; the scale is 0%–0.2% of the total T cell subset for the ICS data and 0–300 sfc/10^6 PBMCs for the ELISpot data. *B*, Median magnitudes of total T cell response at week 4 as measured by the ICS assay (percentage of total $CD4^+$ or $CD8^+$ T cells producing cytokine) and the ELISpot assay (no. of spot-forming cells per 10^6 PBMCs). Subjects are grouped by preimmunization 90% adenovirus serotype 5 (Ad5) neutralization titers (<1:12 or ⩾1:12); at screening, the proportion of subjects with an Ad5 antibody titer <1:12 was 6 (100%) of 6 in the placebo group, 3 (30%) of 10 in the group receiving 10^9 particle units (PUs), 4 (40%) of 10 in the 10^{10}-PU group, and 7 (70%) of 10 in the 10^{11}-PU group. The scale is 0%–0.15% of the total T cell subset for the ICS data and 0–300 sfc/10^6 PBMCs for the ELISpot data.

related to the immunogenicity of EnvC in the vaccine or to enhanced antigenicity of the EnvC protein used in the ELISA. The rAd5 vaccine induced HIV-1 Env–specific antibodies to multiple clades, but the induction of neutralizing antibodies remains an elusive goal.

The existence of neutralizing antibodies to Ad5 in adults has led to questions regarding the utility of rAd5-based vaccines in humans. Nonhuman primate studies have suggested that preexisting Ad5 immunity diminishes the cellular response to vaccine antigens [40] and that higher doses of rAd5 are required to overcome the immune inhibiting effects of preexisting Ad5 immunity. Selected vaccine recipients in the present study with

high levels of Ad5 neutralizing antibody mounted significant HIV-specific humoral and cellular immune responses, indicating that preexisting neutralizing antibodies do not preclude the induction of an immune response to the recombinant antigens expressed by rAd5. However, overall the subjects with preexisting Ad5 immunity had T cell responses that were ~3-fold lower in magnitude than those of the Ad5-seronegative subjects.

The present clinical study identified 10^{10} PUs as a well-tolerated rAd5 dose that can stimulate a multiclade HIV-1 immune response in subjects with a range of preexisting Ad5 antibody titers for further evaluation as a booster vaccine to be used in combination with a multiclade HIV-1 DNA vaccine candidate [17]. Although some vaccine recipients at the 10^{11}-PU dose experienced a short-lived and self-limited syndrome of headache, myalgia, malaise, and fever, they also had better induction of HIV-1–specific antibody and a trend toward better maintenance of T cell responses. Therefore, additional studies evaluating dose are in progress concomitantly with the expanded studies evaluating the 10^{10}-PU dose as a booster vaccine. The present clinical trial demonstrates the safety and immunogenicity of a rAd-based multigene, multiclade HIV-1 vaccine in humans and may represent a step toward the development of a globally relevant vaccine regimen.

VACCINE RESEARCH CENTER (VRC) 006 STUDY TEAM

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